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Two mitochondrial matrix proteases act sequentially in the processing of mammalian matrix enzymes

(protein transport/rat liver/processing peptidases/ornithine transcarbamylase/malate dehydrogenase)

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ABSTRACT **The imported precursors of the mammalian matrix enzymes malate dehydrogenase [(*S*)-malate:NAD$^+$ oxidoreductase, EC 1.1.1.37] and ornithine transcarbamylase (carbamoyl-phosphate:L-ornithine carbamoyltransferase, EC 2.1.3.3) are cleaved to their mature subunits in two steps, each catalyzed by matrix-localized processing proteases. The number and properties of these proteases are the subjects of this report. We have identified and characterized two distinct protease activities in a crude matrix fraction from rat liver: processing protease I, which cleaves these precursors to the corresponding intermediate form; and processing protease II, which cleaves the intermediate forms to mature subunits. Protease I is insensitive to chelation by EDTA and to inactivation with *N*-ethylmaleimide; protease II is inhibited by 5 mM EDTA and is inactivated by treatment with *N*-ethylmaleimide. We have prepared from mitochondrial matrix an 800-fold-enriched protease I fraction free of protease II activity by using the following steps: ion exchange, hydroxyapatite, molecular sieving, and hydrophobic chromatography. Using similar procedures, we also have prepared an approximately 2000-fold-enriched protease II fraction, which has a trace amount of contaminating protease I. This enriched protease II fraction has little or no cleavage activity toward mitochondrial precursors but rapidly and efficiently converts intermediate forms to mature size. Finally, we show that protease I alone is sufficient to cleave the precursor of a third nuclear-encoded mitochondrial protein subunit—the β subunit of propionyl-CoA carboxylase [propanoyl-CoA:carbon dioxide ligase (ADP-forming), EC 6.4.1.3]—to its mature size.**

The vast majority of mitochondrial proteins are encoded by nuclear genes and synthesized as larger precursors with amino-terminal amino acid extensions, which we call leader peptides (1, 2). These peptides, characterized by the presence of basic amino acid residues and the absence of acidic residues, are themselves sufficient to direct mitochondrial uptake of normally cytosolic, as well as mitochondrial, "passenger" proteins (3–5). After translocation of these mitochondrial precursors into the mitochondrial matrix, leader peptides are cleaved from the mature subunit by one or more mitochondrial proteases.

We have been studying mitochondrial protein biogenesis using as a model system ornithine transcarbamylase (OTCase; carbamoyl-phosphate:L-ornithine carbamoyltransferase, EC 2.1.3.3), a homotrimeric mammalian mitochondrial matrix protein of the urea cycle. OTCase is encoded in the nucleus and synthesized on free polysomes as a 40-kDa precursor (pOTCase) bearing a 32-amino acid NH_2-terminal leader peptide (6, 7). This peptide is necessary and sufficient to direct the posttranslational import of the precursor into the mitochondrial matrix by a process requiring a mitochondrial membrane potential (4, 8). During posttranslational import of the rat OTCase precursor into isolated mitochondria, an OTCase species intermediate in size between pOTCase and mature OTCase, called iOTCase, is produced (9, 10). We have shown previously (*i*) that the rate of appearance and rate of disappearance of iOTCase are those of an authentic intermediate in the conversion of pOTCase to OTCase, (*ii*) that rat iOTCase is located in the mitochondrial matrix, and (*iii*) that it is formed by cleavage of the 32-amino acid residue leader peptide between residues 24 and 25 by a matrix-localized processing protease (10). A second cleavage, again catalyzed by a matrix protease, cleaves iOTCase to mature OTCase (10). Three fully processed OTCase subunits then assemble into active OTCase (11). This same pattern of two-step cleavage has been demonstrated recently for a second matrix enzyme precursor, that for rat malate dehydrogenase [(*S*)-malate:NAD$^+$ oxidoreductase, EC 1.1.1.37; MDHase] (12), pMDHase.

The number and properties of the matrix proteases responsible for the two steps of OTCase and MDHase maturation are the subjects of this report. In prior work directed toward isolating a rat liver mitochondrial matrix protease, Mori and coworkers (13–16) were able to purify partially a protease that was reportedly dependent on divalent metal ions and that formed iOTCase from pOTCase. They were unable to recover a mature OTCase-forming activity, however. This led these investigators to suggest that a single matrix metalloprotease was responsible for both cleavages. In contrast, using a crude mitochondrial matrix fraction from rat liver, we repeatedly have detected two functionally distinct proteolytic activities acting on pOTCase. One leads to formation of iOTCase; the second produces mature OTCase.

We report here the characterization and partial purification of these proteases, designated matrix processing protease I and matrix processing protease II. We show that these two activities act sequentially in the removal of the leader peptides from rat pMDHase and from rat and human pOTCase, while only protease I is required for the apparently single step removal of the leader peptide from a third nuclear-encoded matrix polypeptide—the precursor of the β subunit of rat propionyl-CoA carboxylase [PCCase; propanoyl-CoA: carbon-dioxide ligase (ADP-forming), EC 6.4.1.3], pβ-PCCase.

MATERIALS AND METHODS

Synthesis of Precursor Substrates. *In vitro* translation using rabbit reticulocyte lysates was performed as described (17)

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Abbreviations: OTCase, ornithine transcarbamylase; pOTCase, the 40-kDa precursor of OTCase; iOTCase, the 37-kDa intermediate found during import of OTCase; MDHase, malate dehydrogenase; pMDHase, the precursor of MDHase; iMDHase, the intermediate formed during mitochondrial import of MDHase; PCCase, propionyl-CoA carboxylase; β-PCCase, the 54-kDa β subunit of PCCase; pβ-PCCase, the 58.5-kDa precursor of β-PCCase.

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with mRNAs produced by *in vitro* transcription from cDNAs cloned into transcription vectors. Transcriptions with phage SP6 (Promega Biotec, Madison, WI) and phage T7 (United States Biochemical, Cleveland) polymerases were performed according to suppliers' recommendations. Construction of transcription templates for human pOTCase, rat pOTCase, rat pβ-PCCase, and rat pMDHase has been described (10, 18–20).

Preparation of of Mitochondria and Mitochondrial Matrix. Intact mitochondria were isolated from rat livers as described (21, 22). A mitochondrial matrix fraction was prepared essentially as described (3) except that mitoplasts were not prepared; instead, intact mitochondria were solubilized with 0.3 mg of lubrol per mg of protein. Aliquots of each fraction were kept until used at −70°C in 10 mM Hepes buffer (pH 7.4) containing 20 mM NaCl and 0.2 mM of dithiothreitol.

Incubation of the Translation Mixture with Matrix and Purified Processing Proteases. In a typical experiment, 2.5 μl of translation mixture was supplemented with an equal volume of the 100,000 × *g* supernatant from the reticulocyte lysate and incubated with matrix or purified proteases in a final volume of 10 μl. All reactions contained 0.1 mM $ZnCl_2$ and 0.1 mM $MnCl_2$. Products of the reaction were then analyzed directly on $NaDodSO_4$/PAGE. When indicated, lanes were scanned with a scanning densitometer (model SL-DNA, Biomed Instruments, Fullerton, CA). To improve processing of pβ-PCCase, processing proteases I and II were added to the translation mixture at the start of the translation reaction.

RESULTS

Partial Purification of Two Protease Activities from Rat Liver Mitochondrial Matrix. Using ion-exchange chromatography on DEAE-Bio-Gel A, fractionation on hydroxyapatite, elution from heparin-Sepharose, separation on Bio-Gel P-200, and hydrophobic chromatography on ω-aminooctyl-agarose, we separated and partially purified two distinct mitochondrial matrix processing activities, protease I and protease II. Protease I was purified approximately 800-fold and is virtually free of protease II; protease II was purified approximately 2000-fold but is still contaminated with trace amounts of protease I.

A crude mitochondrial matrix fraction from rat liver prepared as described catalyzed cleavage of rat pOTCase to both iOTCase and mature OTCase (Fig. 1*A*, lane 2). Also shown in Fig. 1*A* are the results of incubation of rat pOTCase with either 800-fold-purified matrix protease I (Fig. 1*A*, lane 4) or 2000-fold-purified protease II (Fig. 1*A*, lane 6). Note that the protease I fraction was able to catalyze formation of iOTCase but was devoid of mature OTCase-forming activity. Partially purified protease II generated predominantly mature OTCase; however, a small amount of iOTCase was also seen.

Protease I and Protease II Have Different Sensitivities to *N*-ethylmaleimide. Upon prior incubation of the crude matrix fraction with 2.5 mM *N*-ethylmaleimide, formation of mature OTCase was abolished while iOTCase production was unaffected (Fig. 1*A*, lane 3). Similarly, the production of iOTCase by partially purified protease I was unaffected by *N*-ethylmaleimide treatment (Fig. 1*A*, lane 5), whereas the mature OTCase-forming activity of protease II was abolished by this reagent (Fig. 1*A*, lane 7). Some iOTCase was produced upon incubation of the *N*-ethylmaleimide-treated protease II fraction with pOTCase (Fig. 1*A*, lane 7), almost surely reflecting residual protease I activity in the protease II fraction.

Matrix Processing Protease II but not Matrix Processing Protease I Is Dependent on Divalent Metal Ions. Incubation of a crude mitochondrial matrix fraction with rat pOTCase led to the formation of both iOTCase and OTCase (Fig. 1*B*, lane 2). Incubation in the presence of 5 mM EDTA inhibited formation of mature OTCase but not of iOTCase (Fig. 1*B*, lane 3). Partially purified protease II was as sensitive to 5 mM EDTA as was the protease II activity found in the crude matrix fraction (Fig. 1*B*, lane 7), whereas partially purified protease I was resistant to EDTA treatment (Fig. 1*B*, lane 5).

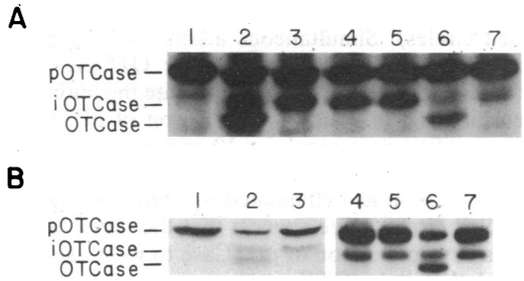

FIG. 1. Inhibition of processing proteases by *N*-ethylmaleimide and EDTA. (A) A crude mitochondrial matrix fraction (lanes 2 and 3) and purified fractions of processing proteases I (lanes 4 and 5) and II (lanes 6 and 7) were incubated for 10 min at 27°C in the absence (lanes 2, 4, and 6) and the presence (lanes 3, 5, and 7) of *N*-ethylmaleimide at a final concentration of 2.5 mM. One volume of the translation mixture containing rat pOTCase was then added, and the incubation was continued for an additional 60 min at 27°C. Lane 1 contains translation mixture only. (*B*) Crude mitochondrial matrix fraction (lanes 2 and 3), purified protease I (lane 4 and 5), and a mixture of purified proteases I and II (lanes 6 and 7) were incubated with translation mixture containing rat liver pOTCase in the absence (lanes 2, 4, and 6) or the presence (lanes 3, 5, and 7) of EDTA at a final concentration of 5 mM for 60 min at 27°C. Lane 1 contains translation mixture only.

Initial Production of iOTCase by Protease I Provides Substrate for the Mature Protein-Forming Protease II. Inhibition experiments such as those shown in Fig. 1 suggest that formation of mature OTCase by fractions enriched for matrix protease II may depend on the prior cleavage of precursor to intermediate, catalyzed by the small amount of residual protease I in these fractions. If this were the case, we would expect formation of mature OTCase by protease II preparations to be more efficient when iOTCase is provided as substrate. To test this hypothesis, we carried out the experiment shown in Fig. 2. The protease II fraction catalyzed a slow but detectable conversion of rat pOTCase to mature

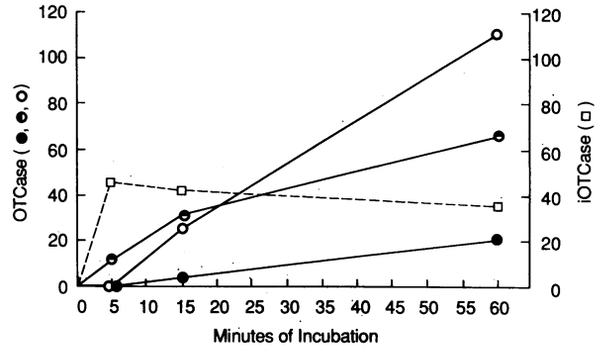

FIG. 2. Time course of conversion of pOTCase to iOTCase and mature OTCase. Translation mixtures containing rat pOTCase were incubated at 27°C for various intervals under conditions shown below. At each time point (5, 15, and 60 min), aliquots were removed and analyzed by $NaDodSO_4$/PAGE; OTCase and iOTCase were quantitated by scanning densitometry of fluorographs and expressed on the ordinates in arbitrary units. ●, Incubation with protease II; ○, incubation with protease I; ⊖, incubation for 10 min with protease I followed by addition of protease II; □, iOTCase formation and disappearance after simultaneous addition of protease I and protease II.

OTCase but only after a characteristic lag of about 5 min (Fig. 2, closed circles). Simultaneous addition of the protease I fraction increased the rate of mature OTCase formation approximately 6-fold but did not eliminate the initial lag (Fig. 2, open circles). In contrast, incubation of pOTCase with partially purified protease I for 10 min prior to addition of partially purified protease II both increased the rate of mature OTCase formation and eliminated the initial lag period (Fig. 2, half-filled circles). We suggest that the initial lag reflects the necessary conversion of pOTCase to iOTCase by protease I and that it is iOTCase that serves as substrate for protease II. Consistent with this explanation, there was indeed an initial burst of iOTCase formation upon incubation of pOTCase with the combined protease I and protease II fractions, followed by disappearance of the intermediate as mature OTCase was produced (see the squares of Fig. 2).

Human pOTCase Is Converted to OTCase via an Intermediate. During *in vitro* import of rat pOTCase by isolated mitochondria, the appearance and disappearance of iOTCase is obvious and easy to detect (10). On the other hand, when the human pOTCase precursor is incubated with these isolated mitochondria, iOTCase is not detected; instead, mature OTCase alone appears after a lag of 5–10 min (18). This could be due to rapid and quantitative conversion of human iOTCase to OTCase so that the intermediate species has too short a half-life to be detected. If this were the case, removal of protease II from a cleavage reaction should lead to accumulation of the previously undetected human iOTCase. Fig. 3, lane 5, shows accumulated human iOTCase produced during a 60-min incubation of human pOTCase with partially purified protease I. Upon addition of the protease II fraction to an aliquot of this reaction mixture, we observed remarkable production of mature OTCase (Fig. 3, lane 6) with concomitant loss of the accumulated intermediate. During a 5-min incubation, no mature OTcase was produced by the protease II fraction alone (Fig. 3, lane 2), and 5 min of coincubation of a protease I fraction and the protease II fraction stimulated mature OTCase production only slightly (Fig. 3, lane 4). Clearly, the primary role of protease II is the conversion of iOTCase to its mature counterpart.

pMDHase also Undergoes Two-Step Cleavage Catalyzed by Protease I Followed by Protease II. It has been shown previously that mitochondria and a crude mitochondrial matrix fraction process rat pMDHase to the mature subunit via an intermediate form (12). To determine whether this similarity to OTCase indicates similar sequential cleavage of pMDHase by protease I followed by protease II, we carried out the experiment shown in Fig. 4. A *N*-ethylmaleimide-treated crude matrix fraction converted 95% of the input pMDHase into an intermediate-size MDHase (iMDHase) without producing any detectable mature MDHase (Fig. 4, lane 2). Similar *N*-ethylmaleimide-resistant conversion was catalyzed by partially purified protease I (data not shown). Upon subsequent incubation of an aliquot of this reaction mixture with partially purified protease II, iMDHase was rapidly converted to mature MDHase (Fig. 4, lane 3). On the other hand, the protease II-enriched fraction alone, when added directly to pMDHase, converted little of the precursor to the mature form (Fig. 4, lane 4). Since most of the input pMDHase was converted to the intermediate during the incubation shown in lane 2, it is clear that the mature MDHase shown in lane 3 must have been produced by cleavage of iMDHase.

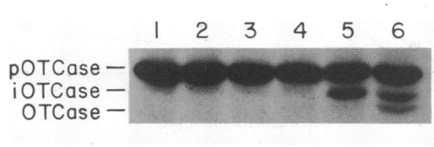

FIG. 3. Two-step conversion of human pOTCase into mature OTCase. Translation mixtures containing human pOTCase were incubated without additions (lanes 1–4) for 60 min at 27°C. Protease I (lanes 1 and 2) or proteases I and II (lanes 3 and 4) were then added to 5-μl aliquots to a final volume of 10 μl. The reaction was either stopped at 0 min (lanes 1 and 3) or continued for an additional 5 min at 27°C (lanes 2 and 4). In the second part of the experiment, translation mixtures were incubated with processing protease I for 60 min at 27°C and then split into two halves. One half was incubated further without protease II (lane 5) and the second half was incubated with protease II (lane 6) for 5 min at 27°C.

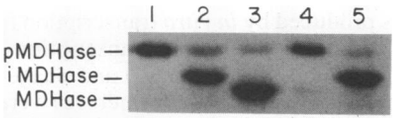

FIG. 4. Conversion of iMDHase to mature MDHase. A crude mitochondrial matrix fraction was incubated with *N*-ethylmaleimide (2 mM) for 10 min at 27°C; dithiothreitol was then added to a final concentration of 3 mM followed by addition of the translation mixture containing pMDHase (lane 1). After incubation for 15 min at 10°C (lane 2), portions were incubated further with (lane 3) or without (lane 5) added protease II for an additional 30 min at 10°C. The translation mixture in lane 4 was incubated with processing protease II alone for 30 min at 10°C.

Protease I Cleaves pβ-PCCase to its Mature Size. To establish which of the two rat liver matrix processing proteases is responsible for the cleavage of leader peptides removed in a single step, we incubated the partially purified proteases with pβ-PCCase. Upon incubation with enriched protease I, pβ-PCCase was efficiently converted to its mature form, β-PCCase (Fig. 5, lane 2). pβ-PCCase was also cleaved by a protease II fraction but to a much lesser extent (Fig. 5, lane 3); this can be accounted for by the amount of residual protease I in this fraction. Incubation of pβ-PCCase simultaneously with the protease I and protease II fractions led to no increase in the production of mature β-PCCase (Fig. 5, lane 4) compared to that catalyzed by protease I. More importantly, there was no change in the size of the β-PCCase produced by the mixture of protease I and protease II fractions (Fig. 5, lane 4).

DISCUSSION

We have demonstrated here that cooperation between two distinct proteolytic activities found in the rat liver mitochondrial matrix is required for the maturation of at least two mammalian mitochondrial matrix proteins. One activity, which we call mitochondrial matrix processing protease I, is insensitive to *N*-ethylmaleimide and cleaves pOTCase and pMDHase to intermediate-size forms. Protease I also appears to cleave at least one other mitochondrial precursor, pβ-PCCase, to its mature size. Mori and coworkers (13–16) have published similar data regarding the range of specificity of an enriched protease fraction derived from rat liver mitochondrial matrix. Their protease fraction cleaved pOTCase to its intermediate form and cleaved five other matrix enzyme precursors to their apparently mature size (16). This suggests that their purified protease may be the same as the one we call protease I. In contrast to the work of Mori and coworkers,

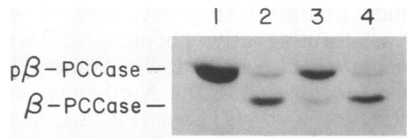

FIG. 5. Cleavage of the β-PCCase. A translation mixture containing pβ-PCCase mRNA and [^{35}S]methionine was incubated in the absence of added protease (lane 1) or in the presence of protease I (lane 2), protease II (lane 3), or both proteases (lane 4).

however, we have not found a divalent metal ion requirement for protease I activity.

The second activity, which we call mitochondrial matrix processing protease II, is sensitive to *N*-ethylmaleimide and cleaves iOTCase and iMDHase to their mature forms. Protease II is sensitive to chelation of divalent cations; in fact, full activity requires supplementation of the reaction mixture with $ZnCl_2$, even in the absence of added chelating agents. Interestingly, we have not yet found a mitochondrial precursor that is converted directly to its mature size by protease II.

We have previously determined the particular residues in the leader peptides of OTCase and MDHase that flank the point of cleavage by protease I (10, 12). This protease cleaves both of these leader peptides at a peptide bond one amino acid residue to the carboxyl side of an arginine residue. We have surveyed the literature and have identified 28 other mitochondrial protein subunits for which the amino terminus of either the mature subunits or of an intermediate resulting from cleavage by a matrix protease is known (J.P.H. and P. Hodges, unpublished data). In 22 of these 28 polypeptides, the leader peptide is cleaved zero to two residues to the carboxyl side of an arginine residue. Thus, it is the initial cleavage of pOTCase and pMDHase by protease I (and not the second cleavage to mature forms by protease II) that is similar to the site of cleavage of most other leader peptides by matrix proteases. We have determined, further, that protease I cleaves at least one precursor directly to its apparent mature size, whereas we have not yet found a precursor that is directly cleaved to its mature form by protease II. For these reasons, we postulate that matrix processing protease I is a general matrix protease that cleaves most, if not all, mitochondrial leader peptides, and that protease II serves to further trim the amino termini of certain species. At this time, we cannot exclude the possibility that protease II has no proteolytic activity of its own but somehow modifies either the specificity of protease I or the conformation of the intermediate substrates so as to allow further cleavage by protease I. Nor can we be absolutely certain that one-step cleavage by protease I, as exemplified by cleavage of pβ-PCCase, gives rise to correct amino termini. Proper evaluation of this hypothesis will require isolation of both proteases in homogenous form and amino-terminal sequence analysis of the products of *in vitro* cleavage by the purified proteases.

The two proteins that we have shown to be acted on by matrix protease II are among the 10 exceptions to the general rule that mitochondrial leader peptides and zero to two amino acids to the carboxyl side of an arginine residue. The amino termini of mature OTCase and MDHase are seven and nine amino acid residues, respectively, to the carboxyl side of the nearest arginine residue in the precursor sequence. We predict that other matrix and inner membrane space proteins with such a mature cleavage site will require a second protease for formation of the correct mature amino terminus. A possible example of such a mitochondrial protein is subunit IV of cytochrome oxidase from *Saccharomyces cerevisiae* (23). The mature amino terminus of this subunit lies four amino acid residues on the carboxyl side of the nearest arginine in the precursor sequence. Significantly, it has been shown that this precursor is cleaved at two distinct sites by intact isolated yeast mitochondria and that a soluble *S. cerevisiae* crude mitochondrial matrix fraction catalyzes cleavage only at the more amino-proximal of these two sites (24). This cleavage occurs eight residues to the amino-terminal side of the mature cytochrome oxidase subunit IV amino terminus and two residues to the carboxyl side of an arginine residue. One possible explanation for these results is that the *S. cerevisiae* protease activity analogous to rat liver protease II is labile and has been lost during preparation of the crude mitochondrial matrix fraction, leaving a processing protease similar to rat liver protease I as the only active matrix protease.

Recently, extensive purification of a matrix processing protease from *Neurospora crassa* has been reported (25). This protease requires divalent metal ions, as assayed by sensitivity to chelators. Two proteins are required for complete processing activity: a 57-kDa protein (which has only weak protease activity on its own) and a 52-kDa protein that enhances this protease activity 10-fold but has no demonstrable activity of its own. A homolog of the 52-kDa protein has been identified by genetic means in *S. cerevisiae* (26). These proteins cannot be detected as a complex and they separate during chromatography under most conditions. Precursors for mitochondrial proteins destined for the matrix, the inner membrane, and the intermembrane space have been tested as substrates for this protease. All precursors tested were cleaved; those precursors tested for which the amino acid sequence surrounding the cleavage site is published were cleaved one to three residues to the carboxyl side of an arginine residue. The cleavage specificity and the range of substrates of the *N. crassa* protease and our rat liver protease I suggest that they may serve homologous functions in these disparate organisms. However, there are significant differences, such as the metal ion requirement and the requirement for an easily separable accessory protein, which cannot be explained at this time. It will be necessary to compare cleavage specificities on identical substrates, antigenic cross-reactivity of the rat liver and *N. crassa* mitochondrial matrix processing proteases, and primary amino acid sequences to clarify the relationship between what we believe must be evolutionarily related protein species.

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