Re-evaluating the Role of His-143 in the Mechanism of Type I Dehydroquinase from *Escherichia coli* Using Two-dimensional $^1$H, $^{13}$C NMR

(Received for publication, October 27, 1997, and in revised form, February 9, 1998)

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Type I dehydroquinase from the shikimate pathway of *Escherichia coli* dehydrates dehydroquinate to dehydroshikimate. pH/log $V_{\text{max}}$ profiles of the enzyme indicate the presence of a single ionizing group with a $pK_a$ of 6.2. Chemical modification experiments with diethyl pyrocarbonate have identified the conserved residue His-143 as essential for catalysis in this enzyme and the $pK_a$ for this modification is also 6.2, implying that this is the single ionizing residue in dehydroquinase that may be acting as a general base in the catalytic mechanism. Subsequent mutagenesis of this residue (Leech, A. P., James, R., Coggins, J. R., and Kleanthous, C. (1995) *J. Biol. Chem.* 270, 25827–25836) further suggested that His-143 may be involved in Schiff base formation/breakdown as well as being the proton abstracting general base. The importance of this residue was confirmed by recent x-ray crystallographic data showing His-143 to be at the center of a hydrogen-bonded triad, flanked by the essential Schiff base forming residue Lys-170 and Glu-86. In the present study, we have used mutagenesis and $^1$H and $^{13}$C NMR to assign the resonance of His-143 and probe its ionization state to define more precisely its role in the mechanism of type I dehydroquinase. Following isotopic enrichment of wild-type and His143A dehydroquinase enzymes with [2–$^{13}$C]histidine, the resonance for His-143 was assigned by comparing their $^1$H, $^{13}$C heteronuclear single quantum correlation NMR spectra. pH titrations revealed that whether in the liganded or unliganded state, His-143 does not ionize over the pH range 6–9.5 and so cannot possess a $pK_a$ of 6.2. The NMR data are consistent with this residue remaining unprotonated at pH values optimal for the activity of this enzyme (pH > 7). The role of His-143 is re-evaluated in light of these and the recent structural data, and an alternative candidate for the $pK_a$ of 6.2 is discussed.

The dehydration of 3-dehydroquinic acid to 3-dehydroshikimic acid is catalyzed by the enzyme 3-dehydroquinase (DHQase; EC 4.2.1.10) and occurs in two metabolic contexts, the biosynthetic shikimate pathway and the degradative quinate pathway. In a biosynthetic background, the enzyme introduces a double bond into the hexane ring of the substrate molecule, which is then converted through several enzymatic steps to produce the central metabolite, chorismic acid. From this precursor, numerous aromatic compounds are made including the aromatic amino acids, ubiquinone and vitamin E. The shikimate pathway is found in all prokaryotes and in lower eukaryotes such as fungi and plants but not in mammals and so is recognized as a source of potential targets for antimicrobial agents and herbicides (1). In a catabolic background, dehydroshikimate is further aromatized to produce protocatechuic acid that goes on to be metabolized to acetyl-CoA through the $\beta$-ketoacylate pathway (2). The quinate pathway, although best characterized in fungi, has also been found in prokaryotes such as *Amylocapsotis methanolica* and *Acinetobacter calcoaceticus* (3, 4).

Two types of dehydroquinase enzyme have been identified, known as type I and type II which do not share any sequence similarities and represent an unusual case of convergent evolution (5). Unlike the serine proteases (the best known example of convergent evolution), which have evolved from different ancestors to yield enzymes that catalyze the same chemical reaction by the same mechanism, dehydroquinases have evolved from different ancestors to catalyze the same chemical reaction by completely different mechanisms. Moreover, the structure and properties of the two types of DHQase are also different. Indeed, the only point of convergence for this class of enzyme is the dehydration reaction itself. It is therefore of great mechanistic and evolutionary interest to study the structure and mechanism of dehydroquinase enzymes to understand how this situation has arisen. Both types have been found in shikimate and quinate pathways, although there remain few examples of a type I DHQase in a quinate background, showing that the metabolic context of the reaction does not play a role in defining which class of DHQase is utilized (6).

Type I enzymes (typified by the enzyme from *E. coli*) are dimers of 25-kDa monomeric molecular mass, are heat labile (denaturing above 60 °C), and catalyze the dehydration of dehydroquinate by a Schiff base mechanism (5). By contrast, type II enzymes (typified by the enzyme from *Anacystis nidulans*) are dodecamers of 16–18-kDa monomeric molecular mass, are very resistant to heat denaturation (>80 °C), and do not catalyze the dehydration reaction by a Schiff base mechanism but...
we have termed “stalled catalysis” (13). This is strikingly illustrated by the fact that the H143A enzyme as purified from overexpressing bacterial cells contains the bound product dehydroshikimate at the active site and this has to be subsequently removed by treatment with hydroxylamine.

These results imply that His-143 is involved in Schiff base formation/hydrolisis as well as possibly playing a role in proton abstraction. Leech et al. (13) hypothesized that it could be involved in both the formation of the Schiff base as a general acid and as a general base in the subsequent proton-abstracting step. The structure for the borohydride-reduced product bound form of type I S. typhi enzyme (which is ~80% identical to the E. coli enzyme; Ref. 16) has recently been solved at 2.5 Å resolution. The structure shows His-143 to lie at the center of a hydrogen-bonded triad linking Lys-170 and Glu-86 (Fig. 1), which is consistent with a role in Schiff base formation but not that originally proposed, rather one in which it deprotonates the Schiff base-forming lysine. However, it remains unclear from the structure if His-143 could go on to participate in general base catalysis in the subsequent elimination reaction.

The ionization state of His-143 is central to our understanding of the mechanism of type I DHQases, and so we set out to determine the ionization behavior of this residue in both unliganded and liganded states. We present 1H and 13C NMR experiments aimed at assigning the resonance of His-143 and which report its ionization state in the active site of the enzyme.

MATERIALS AND METHODS

Chemicals—Ammonium 3-dehydroquinate was prepared by the method of Grewe and Haendler (17). Hydroxylamine hydrochloride was from Sigma. [2-13C]histidine was purchased from Cambridge Isotope Laboratories (Promochem). Other chemicals were of reagent grade or better and used without further purification.

Overexpression, Purification, and Characterization of Wild Type and Mutant 3-Dehydroquinases—These procedures have been described by Leech et al. (13). In all instances protein was determined to be essentially pure by SDS-polyacrylamide gel electrophoresis, and structural integrity of each polypeptide, especially that of the H143A mutant, was verified by electrospray mass spectrometry as described previously (13). Following the purification of H143A DHQase, endogenously bound product was removed by dialyzing the protein against 50 mM potassium phosphate buffer containing 50 mM hydroxylamine followed by a final dialysis against 50 mM potassium phosphate. Borohydride reduction of product at the active site of wild-type DHQase was carried out as described by Leech et al. (13).

Protein Determinations and Enzyme Assays—Protein concentration determinations were carried out by the method of Bradford using a kit from Bio-Rad with bovine serum albumin as a standard (18) and by spectrophotometry using the extinction coefficient ε280 = 19,000 at 280

It is most likely His-143, the role of which is to abstract the C2 proton of the substrate following Schiff base formation at the active site residue (13). The activity of the H143A mutant, producing burst kinetics under steady-state conditions, the H143A mutant is thus able to slowly convert substrate to product but is severely hampered in its ability to hydrolyze the product Schiff base, a phenomenon that has been determined for this enzyme from pH/log K values which is syn for the E. coli type I enzyme (8) but anti for the A. nidulans type II enzyme (9). Differing crystals of both types of DHQase have been obtained, the type II enzyme from Mycobacterium tuberculosis and the type I enzyme from Salmonella typhi (10, 11).

Several chemical modification and mutagenesis experiments have been conducted on the type I DHQase that reveal some features of its mechanism and protein chemistry. The Schiff base-forming lysine has been identified as Lys-170 (12), which is highly conserved in this class of enzyme. The role of the Schiff base is to act as an electron sink following proton abstraction of the C2 proton. The Schiff base may also play a role in distorting the carbocyclic ring of dehydroquinate to render the C2 proton more reactive (9). Mutagenesis reveals that although a covalent adduct is formed between the enzyme and the ligand, this carbanion stabilizing moiety plays little or no role in ground state binding of substrate or product but is purely a catalytic device (13).

Diethyl pyrocarbonate readily inactivates the type I enzyme from E. coli due to the modification of a single histidine residue (14). Although several histidine residues were modified by this reagent, the conserved residue His-143 or the neighboring nonconserved His-146 were identified as the site of inactivation. Subsequent mutagenesis of each confirmed that the conserved His-143 is essential for the catalytic mechanism, whereas His-146 does not play a role (13). Deka et al. (14) also found that the diethyl pyrocarbonate inactivation was very pH-dependent exhibiting a single pK of 6.18, which is identical to the single pK that has been determined for this enzyme from pH/log Vmax profiles (15). The two sets of data suggest that the type I enzyme from E. coli contains a general base in the active site, most likely His-143, the role of which is to abstract the C2 proton of the substrate following Schiff base formation at Lys-170.

His-143 has taken on a more enigmatic role following the characterization of the mutant in which this residue had been substituted for alanine (13). This is strikingly illustrated by the fact that the H143A enzyme as purified from overexpressing bacterial cells contains the bound product dehydroshikimate at the active site and this has to be subsequently removed by treatment with hydroxylamine.

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nm (14). Dehydroquinase activity was assayed by monitoring the appearance of product by spectrophotometry at 234 nm as described previously (15).

Incorporation of [2-\textsuperscript{13}C]Histidine into Wild-type and Mutant Dehydroquinases—The plasmids pAL2, pAL50, and pAL7 expressing wild-type E. coli DHQase and H146A DHQases, respectively, were transformed into E. coli strain ATC1360 (A/gpt-proa/62, lacY1, tex-29, supE44, galk2, λ-,aro D6, his G43oc), xyl-5, mtl-1, arg E3, thi-1) obtained from the E. coli genetic stock center. A 50-ml seed flask of M9 minimal medium supplemented with arginine, proline (100 µg/ml), [2-\textsuperscript{13}C]histidine (40 µg/ml), thiamine (10 µg/ml), and ampicillin (200 µg/ml) was inoculated from a glycerol stock and grown for approximately 24 h. For the H143A protein, the medium was also supplemented with 200 µg/ml shikimic acid. This culture was then used to inoculate 500-ml flasks of the same medium, which were grown to about 18 h. The labeled protein was purified as described previously (15).

Preparation of NMR Samples and NMR Parameters—Protein purified by fast protein liquid chromatography was dialyzed against 1 mM potassium phosphate buffer (pH 7.0) and lyophilized to dryness. The powder was redissolved in potassium phosphate buffer made up in D\textsubscript{2}O to give final protein concentrations of about 6–8 mg/ml and 50 mM phosphate. Samples were centrifuged briefly before NMR experiments. pH titrations were performed using small quantities of KOD or DCl in D\textsubscript{2}O, generally toward alkaline pH values to avoid precipitation; pH values were determined directly in the NMR tubes using a combination of 

RESULTS AND DISCUSSION

\textsuperscript{1}H-NMR Studies, Assignment of His-146—NMR is ideally suited to the determination of residue pK\textsubscript{a} values in proteins (22). Since the C\textsubscript{2} proton of histidine residues lies in the aromatic region of \textsuperscript{1}H NMR spectra it is sometimes possible to follow the ionization changes of a histidine residue by observing this signal without resorting to isotope enrichment methods. At 55 kDa, the type I DHQase dimer is a large protein by NMR standards but successful determinations of histidine pK\textsubscript{a} values for proteins of this size have been reported previously (24). Samples of type I DHQase were dialyzed into D\textsubscript{2}O/phosphate buffer (as described under “Materials and Methods”) and one-dimensional \textsuperscript{1}H spectra recorded at 600 MHz (Fig. 2). The aromatic region of the wild-type spectrum indicates many sharp and broad resonances superimposed on a bank of nonexchanged amide protons. To assign the resonance for the essential active site histidine residue His-143, we compared the wild-type spectrum to that of the H143A mutant (Fig. 2, bottom and middle, respectively). In addition, we also analyzed samples of the H146A mutant (which is fully active) as a control (Fig. 2, top). Histidine C\textsubscript{2} protons reside downfield of the bulk of the aromatic resonances (6.5–7.5 ppm). No clear differences could be seen however for H143A in this region by comparison with the wild-type protein but an unambiguous assignment could be made for the sharp resonance at 7.9 ppm as that from His-146. pH titrations of these samples (over the range 6–8) also failed to provide any evidence for the loss of a peak in the H143A mutant relative to the wild-type enzyme but did reveal that His-146 in type I DHQase has a pK\textsubscript{a} of 6.86 ± 0.02 (data not shown). The significance of this observation is discussed later. Ligand binding can perturb residue chemical shifts and so one-dimensional \textsuperscript{1}H spectra were obtained for wild-type, H143A, and H146A following covalent attachment of the product dehydroshikimate to the Schiff base-forming lysine by borohydride reduction (25). Whereas some perturbations could be seen in the wild-type spectrum, identical changes were observed in the mutant spectra (data not shown). Thus, even after irreversible attachment of product to the active site, the \textsuperscript{1}H resonance for His-143 could not be obtained by comparison to the alanine mutant.

Isotopic Enrichment, \textsuperscript{1}H,\textsuperscript{13}C-HSQC NMR Experiments and Assignment of His-143—Isotopic enrichment of proteins with NMR isotopes, \textsuperscript{15}N or \textsuperscript{13}C, makes the identification and study of individual residues in large proteins tractable and useful approach when attempting to define their ionization state (26). \textsuperscript{15}N, for example, has been particularly useful in the analysis of histidine residues in the catalytic triad of serine proteases (27) and triosephosphate isomerase (28). \textsuperscript{13}C NMR has also been used to study proteins such as the serine proteases (29). Even
for a protein as large as the catalytic subunit of aspartate transcarbamoylase (100 kDa), information on the ionization state of an active site histidine residue was obtained by $^{13}$C amino acid enrichment in combination with $^{13}$C NMR (30).

In the present study, type I DHQase was enriched with $^{13}$C2-labeled histidine using *E. coli* ATC1360, a bacterial strain auxotrophic for histidine (see “Materials and Methods”). Preliminary studies demonstrated that the level of histidine required to establish a good yield of cell paste and achieve a high level of expression on induction with isopropyl-1-thio-β-D-galactopyranoside in defined medium was 40 μg/ml. By comparison with the normal levels of expression that are achieved using *E. coli* ATC1360 in rich medium (100 mg/liter culture), the yields on minimal medium under these conditions were 20 mg/liter culture. Wild-type and both histidine mutants (H143A and H146A) were isotopically enriched by this procedure, concentrated by ultrafiltration, and dialyzed into phosphate buffer. One problem that was encountered in these studies was the limited solubility of the enzyme it does not have a pKa modification experiments of Deka et al. (14), which showed unambiguously that the modification of His-143 inactivates the enzyme and this modification can be described by a single pKa of 6.2. Our NMR data on the unliganded enzyme therefore implies that the ionization observed in the chemical modification experiments may be that of a neighboring residue.

**Effect of Ligand Binding on the Ionization State of His-143**—There are several well documented examples in the literature where ligand binding alters amino acid side chain pKa values in enzyme active sites. In ribonuclease A, for example, the pKa values for the 2 essential histidine residues, which act as a general acid and as a general base, are each raised by nucleotide binding so that they acquire the appropriate charge state for catalysis to proceed (23). We therefore investigated the effect of ligand binding on the $^{13}$C-labeled histidine titration curves of DHQase (Fig. 4B). To avoid the complexities of equilibrium binding we irreversibly linked the product dehydroshikimate to the active site of wild-type DHQase by borohydride reduction (see “Materials and Methods”). Fluorescence spectroscopy and circular dichroism measurements suggest that reduction of the product in this way does not cause any global conformational changes, but rather causes a large change in the stability of the protein (25). The six histidine residues of each DHQase monomer are scattered throughout the protein, and since histidine pKa values are sensitive probes of their electrostatic microenvironment their behavior can also serve as indicators of changes in conformation. The titration behavior of the 6 histidine residues of wild-type DHQase following the irreversible attachment of product reinforces the view that this treatment does not cause a profound change in the conformation of the enzyme since none of the 4 titrating residues are perturbed by more than 0.1 of a pKa unit; the pKa for His-146, for example, in the liganded state is 6.84, which is essentially identical to the unliganded enzyme (6.82).

The only significant change that is observed as a result of the borohydride reduction of product at the active site of wild-type DHQase is that the $^{13}$C chemical shift of His-143 moves downfield by 0.6 ppm (whereas its $^1$H chemical shift moves downfield by 0.16 ppm). It is clear from these data that ligand binding does not cause the pKas of His-143 to rise, and indeed from its chemical shift location, the pKa for this residue is likely to be even lower in the liganded state.

**The Role of His-143 in the Mechanism of Type I DHQase and the Identity of the Residue with a pKa of 6.2—**His-143 has been a strong candidate for the single ionizing group in type I DHQase with a pKa of 6.2 (14, 15). The NMR data presented in this paper show conclusively that this ionizing group is not His-143. Indeed, none of the histidines in *E. coli* type I DHQase has a pKa of 6.2; the closest is His-146 which has a pKa of 6.8 in the presence or absence of ligand. Our data are consistent with a much lower pKa for His-143, likely to be <5.5 both in the liganded and unliganded states, which cannot be defined unambiguously due to problems of precipitation at low pH values. This result demonstrates that His-143 must be unprotonated at pH values at which the enzyme is most active (pH > 7). Since His-143 does not have a pKa of 6.2, and neither do any

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**13C NMR Studies of Type I DHQase Histidine Residues**

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**pH Titrations of $^{13}$C-Labeled Histidine Resonances in Wild-type DHQase—**To obtain complete ionization curves for histidine residues, pKa values that normally range from 4 to 8, a wide spectrum of pH values should be sampled. However, at the concentrations used for the NMR experiments, type I DHQase precipitates readily at pH levels below 6 and so this represents the lowest achievable pH. pH titrations for the enzyme therefore encompassed the pH range 6–9.5, which would nevertheless detect the ionization of a residue with a pKa of 6.2, the predicted pKa for His-143 (14). The pH titrations for the $^{13}$C resonances of labeled wild-type DHQase are shown in Fig. 4A (very similar data were observed for the $^1$H resonances and so are not shown). The data demonstrate that His-143 does not titrate in the pH range 6–9.5 and so in this form of the enzyme it does not have a pKa of 6.2. Moreover, 4 of the 6 histidine residues in unliganded type I DHQase titrate in this pH range (2 of which, His-146 and peak b, were more or less complete titrations), but none of these displayed a pKa of 6.2 either. Each of the titrating peaks was fitted to a single ionizing curve described by the Henderson-Hasselbach equation; the pKa values that were determined were as follows: His-146, 6.84 (±0.05); peak a, 8.72 (±0.08); peak b, 7.61 (±0.04); peak c, 8.00 (±0.07). As for His-143, peak d did not titrate over the pH range studied. The conclusion from these data is that unliganded type I DHQase does not possess a histidine residue with a pKa of 6.2. This is at variance with the diethyl pyrocatechol modification experiments of Deka et al. (14), which showed unambiguously that the modification of His-143 inactivates the enzyme and this modification can be described by a single pKa of 6.2. Our NMR data on the unliganded enzyme therefore implies that the ionization observed in the chemical modification experiments may be that of a neighboring residue.

The site of the $^{13}$C-label in our experiments was at the C2 position, which is ideally placed to signal changes in ionization state. C4-labeled $[^{13}$C]histidine is also a good probe of histidine ionizations. In combination with proton decoupling to reduce line broadening, direct detection of the $^{13}$C nucleus is possible with either probe. However, indirect detection of a $^{13}$C nucleus coupled to protons offers a very sensitive method for observing such sites in proteins. We therefore compared $^1$H,$^{13}$C HSQC spectra of wild-type with both H146A DHQase (Fig. 3A) and H143A DHQase (Fig. 3B) in an attempt to assign their resonances. Wild-type DHQase has 6 histidine residues and from the appropriate region of the spectrum (7–9 ppm, $^1$H dimension; 134–136 ppm $^{13}$C dimension) it can be seen that six resonances are clearly identified, the strongest of which corresponds to His-146, as determined from the one-dimensional $^1$H experiments (Fig. 2). Each of the HSQC spectra for the histidine mutants shows the loss of one cross-peak in comparison with the wild-type spectrum conducted at the same pH. The resonance for His-143 (at 7.85 ppm in the $^1$H dimension and 136 ppm in the $^{13}$C dimension; Fig. 3B) was the weakest in the spectrum and at certain pH levels was almost obliterated by the titrating resonance of His-146. This explains why we could not detect the loss of His-143 in the $^1$H experiments. These data therefore assign the $^{13}$C2-$^1$H cross-peak from the active site histidine, His-143.
of the other histidines in type I DHQase, this ionization constant must be associated with another residue. The fact that this $pK_a$ was measured by chemical modification of His-143 suggests that a residue close to this histidine may be responsible. X-ray crystallography shows His-143 to be part of a hydrogen-bonded triad involving the Schiff base-forming lysine, Lys-170, and Glu-86 (Fig. 1), and so the question arises as to whether either of these residues could have a $pK_a$ of 6.2. The $pK_a$ of a lysine side chain is normally 10.4–11.1 (31). This presents a formidable problem for Schiff base-forming enzymes which must reduce the $pK_a$ of the $\varepsilon$-amino group to enable nucleophilic attack of the substrate carbonyl. In the enzyme acetoacetate carboxylase, for example, this is achieved by placing another positively charged residue nearby that lowers the $pK_a$ to 6.0 (32). It is possible that the $pK_a$ of 6.2 that has been measured for type I DHQase is that of Lys-170 itself.

Although the protein concentrations and number of acquisitions were the same in all experiments, problems of turbidity arose in the $B$ samples, hence the poorer signal-to-noise ratio relative to $A$. The slight changes in chemical shift between some of the peaks in $A$ and $B$ are due to the difference in pH between the two experiments.
and at the present time this cannot be discounted. However, circumstantial evidence suggests that this may not be the case. Our earlier work found that when mutating (singly) Lys-170, His-143, and His-146 to alanine only the H146A mutation significantly affected the isoelectric point of the enzyme, which could be accurately measured by isoelectric focusing gel electrophoresis (13). The pI of type I DHQase is 4.9 for the native enzyme (which migrates as a dimer on native isoelectric focusing gels and does not show signs of precipitation at the low concentrations of protein used) and decreases to 4.6 following mutation of His-146 to alanine. This indicates that at its isoelectric point the enzyme, His-146 is positively charged since removing it causes the enzyme to become more acidic, which is consistent with the available data, clearly requires further analysis.

A logical role then for the lysine residue that, although consistent with the available evidence that its pKᵣ of 6.8 that has been measured by ¹H and ¹³C NMR. The pKᵣ for His-143 is likely to be <5.5, which explains why the pI of the enzyme does not change on making the alanine mutation since it does not carry a full charge or is uncharged. Since the K170A mutation also does not affect the pI of the enzyme it too is likely to be either uncharged or only partially charged at the isoelectric point of the enzyme, implying that its pKᵣ is also ≲5.5. This is an unusually low pKᵣ for a lysine residue that, although consistent with the available data, clearly requires further analysis.

The location of His-143 next to the ε-nitrogen of Lys-170 argues strongly for a role in Schiff base formation/hydrolysis. Contrary to earlier expectations, its distance from the C2 position of the ligand (albeit the product) suggests that it may not be involved in the general base-catalyzed proton abstraction of the pro-R proton. Thus, although His-143 is clearly involved in imine formation and breakdown, its subsequent involvement in the dehydration is uncertain. The properties of the H143A mutant are consistent with this role since the mutant is compromised in its ability to hydrolyze the product Schiff base that accumulates slowly at the active site (13). A logical role then for His-143 is to deprotonate the ε-NH₂ group of Lys-170, which would leave the imidazole group carrying a positive charge. However, since the NMR data show that His-143 is unlikely to carry a positive charge this suggests that the abstracted proton could be further shunted along the triad to Glu-86. It seems reasonable then that Glu-86 may possess the pKᵣ of 6.2 that has been identified for type I DHQase by diethyl pyrocarbonate modification and pH/log Vₘₐₓ profiles.

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*J. Biol. Chem.* 1998, 273:9602-9607.  
doi: 10.1074/jbc.273.16.9602

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