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Effects of pH on kinetics of the structural rearrangement that gates the electron-transfer reaction between zinc cytochrome *c* and plastocyanin. Analysis of protonation states in a diprotein complex*

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Abstract: Electron transfer from zinc cytochrome *c* to copper(II)plastocyanin in the electrostatically-stabilized complex [Crnogorac MM, Shen C, Young S, Hansson O, Kostić NM (1996) *Biochemistry* 35, 16465–74]. We study this rearrangement in four complexes Zncyt/pc(II), which zinc cytochrome *c* makes with the wild-type form and the single mutants Asp42Asn, Glu59Gln, and Glu60Gln of plastocyanin. The rate constant for the rearrangement, k_F, differs for the four forms of plastocyanin but is independent of pH from 5.4 to 9.0 in all four cases. That k_F is affected by the single mutations but not by pH changes suggests that the residues Asp 42, Glu59, and Glu60 in the wild-type plastocyanin remain deprotonated (*i.e.*, as anions) within the Zncyt/pc(II) complex throughout the pH range examined. This fact agrees with the notion that loss of salt bridges in the initial (redox-inactive) configuration of the complex is compensated by formation of new salt bridges in the rearranged (redox-active) configuration.

Keywords: plastocyanin, zinc cytochrome *c*, pH effects, gated electron transfer, site-directed mutagenesis, protein-protein rearrangements.

INTRODUCTION

Metalloproteins act as electron carriers in many biological processes, such as photosynthesis and respiration. Despite much recent research,[1–7] recognition and electron transfer between metalloproteins is only partially understood. A pair of metalloproteins can form multiple complexes in solution, and configuration that is optimal for recognition need not be optimal for electron transfer.[8–17] The proteins may rearrange from the binding configuration to the reactive configuration. If this rearrangement is the rate-limiting step, the electron-transfer reaction is said to be gated.[7]

The heme protein cytochrome *c*[18–20] and the blue-copper protein plastocyanin[21–26] are well suited for kinetic and mechanistic studies since their three-dimensional structures

* Dedicated to Professor Miroslav J. Gašić on the occasion of his 70th birthday.

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in both crystalline and dissolved states are known; they are practically the same in the two states. At pH 7.0 copper(II)plastocyanin has a charge of –8 while iron(II)cytochrome *c* has a charge of +6. The negative charge in plastocyanins from high plants is found mostly in the acidic patch, which consists of the lower cluster – residues Asp 42, Glu 43, and Glu 44 – and the upper cluster – residues Glu59, Glu60, and Asp 61 (numbering from Spinach protein). This patch has been implicated in reactions of plastocyanin with small inorganic complexes and with other proteins.[21,27–32] Some of these acidic residues seem to have unexpectedly high pK_a values. From our previous study it is clear that three of the acidic residues have pK_a above 6.0.[33]

The replacement of iron(II) with zinc(II) does not perturb the conformation of cytochrome *c* and its association with other proteins.[34–37] The zinc(II) derivative, designated Zncyt, is excited by the laser pulse to its triplet state, ^{3}Zncyt, a strong electron-donor (E^0 = –0.88 V *vs*. SHE) that reduces copper(II)plastocyanin. The reaction in Eq. (1) is gated by a structural rearrangement of the electrostatically-stabilized complex ^{3}Zncyt/pc(II). The first-order rate constant k_F corresponds to this rearrangement, not the electron transfer:[37–39]

$$^3\text{Zncyt/pc(II)} \xrightarrow{k_F} \text{Zncyt}^+\text{/pc(I)} \quad (1)$$

Variations in ionic strength, viscosity, thermodynamic driving force, temperature, and site-directed mutation revealed much, but not everything, about the mechanism of this rearrangement.[27,38–42] Both the effects and non-effects (*i.e.*, both the changes and lack of changes) proved informative in these previous studies. Here we examine the effects of neutralizing certain residues in the acidic patch. Besides the wild-type form, we chose the following three mutants of spinach plastocyanin: Asp42Asn, Glu59Gln, and Glu60Gln. The kinetics results reveal the protonation state of these residues in the dynamic diprotein complex.

MATERIALS AND METHODS

Chemicals

Distilled water was demineralized to resistivity greater then 16 MΩ cm. Chromatography gels were purchased from Sigma Chemical Company. Triethanolammonium chloride was purchased from Aldrich Chemical Company. Hydrogen fluoride, nitrogen, and ultrapure argon were purchased from Air Products Co. All other chemicals were purchased from Fisher Chemical Company.

Buffers

The buffers kept the ionic strength constant at 2.50 ± 0.10 mM over the entire pH range, from 5.4 to 9.0.[43] The full range was covered with a buffer made by dissolving 2.50 mmol (0.4641 g) of triethanolammonium chloride and 2.50 mmol (142.8 μL) of glacial acetic acid in 1.000 L of water. The pH interval from 5.4 to 7.2 was covered also with a 2.5 mM solution of sodium cacodylate. The pH interval from 6.6 to 9.0 was covered also with a 2.50 mM solution of triethanolammonium chloride. The basic componenet of the first two buffers and the acidic component of the third were created by adding 0.100 M solutions of NaOH or HCl, respectively. These two solutions were used also to adjust the pH, which was measured with a Fisher Accumet 805 MP pH meter equipped with an Aldrich combination microelectrode. The k_F values in the overlapping region, from 6.6 to 7.2, were the same regardless of the buffer chosen.

Proteins

Cytochrome *c* from horse heart was purchased from Sigma Chemical Co. The iron-free (so-called free-base) form was made, purified, and reconstituted with zinc(II) by a modification[44] of the original procedure.[34,45] The product, zinc cytochrome *c*, was handled at 4 ºC, in the dark. The criteria of purity were the absorbance ratios $A_{423}/A_{549} > 15.4$ and $A_{549}/A_{585} < 2.0$. The absorptivity is $\varepsilon_{423} = 2.43\times10^5$ mol^{-1} dm^3 cm^{-1}.[45] Wild-type plastocyanin from spinach and three single mutants were prepared by overexpression in *E. coli* with the vector pUG223t$_r$[46] and purified first with a DE32 column and then with a 26/10 Q Sepharose high-performance FPLC column from Pharmacia. The blue fraction was concentrated by dialysis against dry polyethyleneglycol (PEG 20000) and passed through a gel-filtration column Sephacryl S-100 HR. The amount of holo-plastocyanin was determined spectrophotometically, in the presence of $K_3[Fe(CN)_6]$, on the basis of the absorptivity ε_{597} = 4700 mol^{-1} dm^3 cm^{-1}.[46] The UV-vis spectra were recorded with a Perkin-Elmer Lambda 18 spectrophotometer.

Flash kinetic spectrophotometry

The so-called laser flash photolysis on a microsecond time scale was done with a standard apparatus.[47] Argon for deaeration was first passed through water and then through the buffered solution of the proteins for 30 min. The cell jacket was connected to the 30-L circulating bath Forma 2067, which maintained the temperature at 293.0(2) K. Concentration of Zncyt was kept at 10.0 μM, while concentration of copper(II)plastocyanin was varied from 2.0 to 30.0 μM. Initial volume of the solution was 1.000 mL. Upon each addition of a small portion, 1.00 to 4.00 μL, of a 0.100 M solution of NaOH or HCl, the pH was measured with the

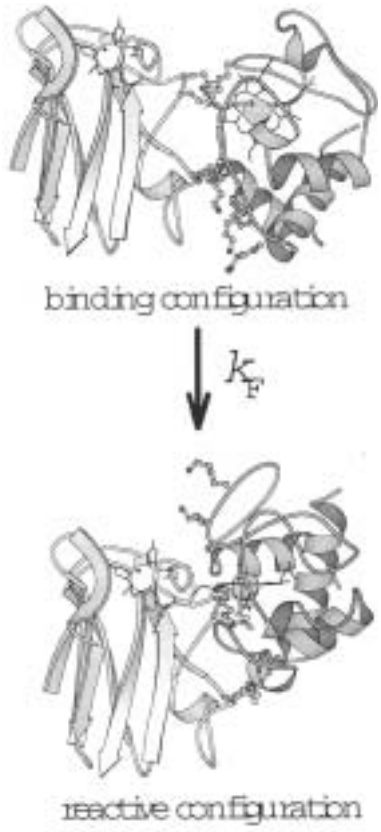

Fig. 1. Rearrangement of the diprotein complex Zncyt/pc(II) from the binding to the reactive configuration, corresponding to the so-called max-ov and n/eq structures found in a computational study.[48] The two metals are highlighted, while the porphyrin ring and the ligands to copper are shown as wire-frame models. Plastocyanin (on the left) is stationary, while cytochrome *c* (on the right) moves. Protein structures were drawn with the program MolScript v2.1.[65]

freshly-calibrated pH meter, and the solution was additionally deaerated for 10 more min. Formation and decay of the triplet state, ^{3}Zncyt, was monitored by change in absorbance at 460 nm, ΔA_{460}. At each concentration of copper(II)plastocyanin and each pH value, four or more laser pulses were delivered and kinetic traces recorded. Calibration of the pH meter was checked after the last adjustment of pH.

Regression analysis of the rate constant k_F

The kinetic traces were fitted to Eq. (2) by a least-squares nonlinear regression method, with the program SigmaPlot v4.01, from SPSS Inc.

$$\Delta A_{460} = a_1 \exp(-k_F t) + a_2 \exp(-k_{obs} t) \quad (2)$$

The pseudo-first order rate constant k_{obs} depends on the concentration of copper(II)plastocyanin and corresponds to bimolecular quenching of ^{3}Zncyt by copper(II)plastocyanin.[33] We focus on the first-order rate constant, k_F, which corresponds to unimolecular quenching in the complex ^{3}Zncyt/pc(II). The error margins for all reported values correspond to two standard deviations and the confidence limit grater than 95 %.

Modeling of the rearrangement

The rearrangement involves the migration of Zncyt on the plastocyanin surface from the binding configuration, in which the basic patch in Zncyt abuts the acidic patch in plastocyanin, to the reactive configuration, in which Zncyt sits near the upper edge of the acidic patch or between the acidic and hydrophobic patch in plastocyanin; see Fig. 1.[38,39,41,42] The respective models for the two configurations are so-called max-ov and n/eq complexes found in a thorough computational search.[48] Our recent studies showed the changes in configuration to be facile, across low barriers.[39,41,42] The rearrangement of the Zncyt/pc complex was modeled and viewed with the program RasMol v2.4.

RESULTS

Redox quenching of ^{3}Zncyt by wild-type copper(II)plastocyanin and its mutants

The rate constant for natural decay of the triplet ^{3}Zncyt remains 80 ± 10 s^{-1} over the pH range, from 5.4 to 9.0.[37,49,50] In the presence of the copper(II)plastocyanin, this excited state is oxidatively quenched. As the concentration of copper(II)plastocyanin increases the rate of the triplet decay increases as well. The rate of triplet decay is mostly biphasic throughout the pH range. The so-called faster phase (first term in Eq. (2)) corresponds to the unimolecular reaction within a preformed ^{3}Zncyt/pc(II) complex, while the so-called slower phase (second term in Eq. (2)) corresponds to the bimolecular reaction between ^{3}Zncyt and pc(II). The effects of pH on the later reaction have been the focus of our previous study,[33] here we focus on the properties of the Zncyt/pc(II) complex and study the unimolecular phase.

As the copper(II)plastocyanin concentration increases, the amplitude a_1 increases, but the rate constant k_F remains the same. At a constant copper(II)plastocyanin concentration, a_1 depends on pH (see Fig. 2), but k_F does not (see Fig. 3). For sufficiently accurate determination of k_F, the concentration of Zncyt/pc(II) complex should be greater than *ca.*, 1.0 μM. Because the mutants Glu59Gln and Glu60Gln have diminished affinity for Zncyt, ewen 30.0 μM concentration of them could not produce a sufficient concentration of Zncyt/pc(II) at low and high ends of the pH range. For this reason, the plots in Figs. 3c and 3d are a little shorter than the others. All four plots in Fig. 3 span the pH ranges over which k_F could be deter-

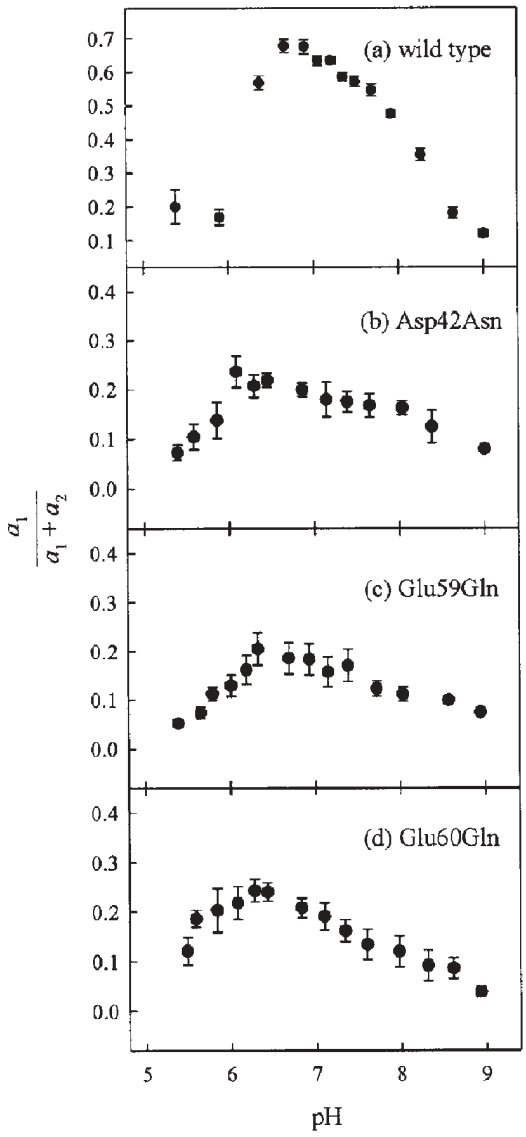

Fig. 2. Dependence on pH at the constant ionic strength of 2.50 mM of the relative amplitude of the unimolecular phase (structural rearrangement) in the reaction in Eq. (1). Concentrations of Zncyt and (a) wild type plastocyanin, (b) Asp42Asn, (c) Glu59Gln, and (d) Glu60Gln mutant forms of plastocyanin were 10.0 μM each.

mined well. The main result, shown in Table I and Fig. 3, is that the rearrangement of the complex Zncyt/pc(II) is unaffected by pH changes.

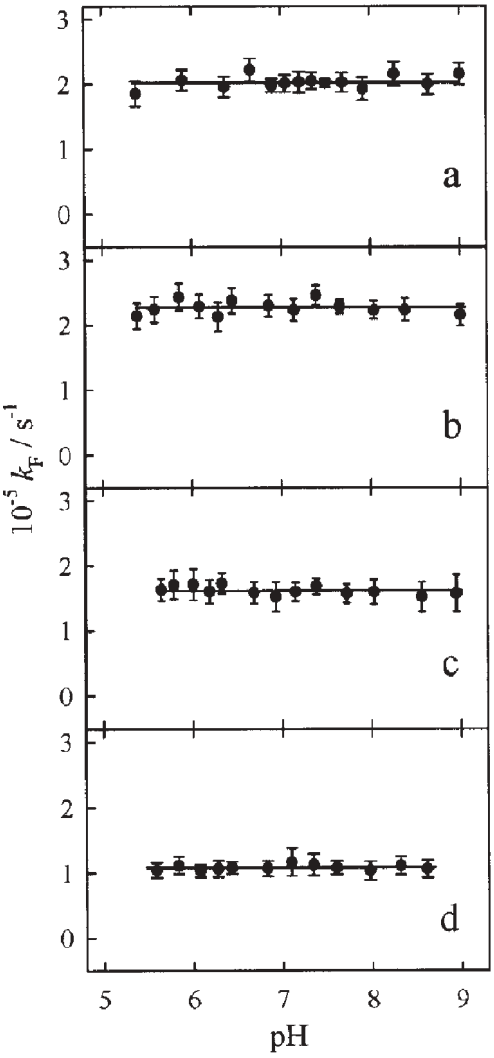

Fig. 3. Independence of pH of the rate constant k_F for the structural rearrangement of the di-protein complex ^{3}Zncyt/pc(II) containing four forms of copper(II)plastocyanin: (a) wild-type, (b) Asp42Asn, (c) Glu59Gln, and (d) Glu60Gln. Each line is the fit to the slope of zero and had $R^2 \geq 0.97$.

TABLE I. The rate constant k_F for the unimolecular phase (structural rearrangement) in the reaction in Eq. (1). The concentration of Zncyt is 10.0 μM, temperature is 293.0 K, and ionic strength is 2.50 mM. The error margins correspond to two standard deviations and the confidence limit greater than 95 %

Plastocyanin form	$10^{-5}\ k_F$ /s^{-1}
Wild-type	2.0(1)
Asp42Asn	2.3(1)
Glu59Gln	1.6(1)
Glu60Gln	1.1(1)

DISCUSSION

Varying the pH

The pH interval from 5.4 to 9.0 is as wide as was safely possible. It could not be extended on the acidic side because protonation of His87 in copper(I)plastocyanin ruptures its bond to the copper(I) atom and prevents its oxidation.[51] The pH interval could not be extended to the basic side because of the possible nonspecific damage to the proteins. Because association of Zncyt and plastocyanin depends on ionic strength, we kept it constant while varying pH.[43]

The reduction potential of the proteins depend only slightly on pH.[18,23,52] The change of 0.04 V or less is negligible in comparison with the driving force of 1.2 V for the photoinduced reaction in Eq. (1). Since this reaction is gated, the rate constant for protein rearrangement, k_F, is independent of the driving force.[40] Because a_1 and k_F are independent of the buffer (at a given pH value), we rule out specific interactions between the proteins and the buffer.

Changes of reaction amplitude with pH

As seen in Fig. 2, when concentration of copper(II)plastocyanin is kept constant and pH is raised from 5.9 to 9.0, the amplitude of the unimolecular reaction increases, reaches a maximum and then decreases. The amount of preformed diprotein complex, Zncyt/pc(II), increases, reaches a maximum, and then decreases which is in direct correlation with the association constant of these two proteins.[33]

Independence of the protein rearrangement of pH

There are relatively few studies of the effects of pH on redox reactions and accompanying processes involving metalloproteins.[53–56] The rate constant for a true electron-transfer reaction within ascorbate oxidase is independent of pH.[57] We, however, know of only one previous study of a gated electron-transfer reaction (*i.e.*, of the gating rearrangement) at various pH values.[56]

Since the pK_a values of three residues in free wild-type plastocyanin are around 6.3–6.4,[33] initially we were surprised by the finding in Fig. 3a, that the rate of rearrangement is independent of pH in an interval reaching well below this value. Then, the second look at the pK_a values of these residues made us realize that in our case pc(II) is in the complex with Zncyt and the pK_a values of these residues would be slightly lower (between 5.8 and 5.9).[33] Spanning the pH range from 5.4 to 9.0 includes these pK_a values as well, but the rearrangement rate constant k_F does not depend on pH in this range.

Such interesting behavior (this independence) may have three explanations, depending on the (de)protonation state of those side chains that are affected by protein association. First, this state changes but is irrelevant for the rate of rearrangement. Second, this state changes but multiple changes cancel one another so that the plot in Fig. 3a remains horizontal. Third, this state does not change.

To distinguish among these three possible explanations, we compared the behavior of three complexes, those that Zncyt makes with the wild-type plastocyanin and with the mu-

tants Asp42Asn and Glu59Gln; see Figs. 3a, 3b, and 3c. Carboxylic acid and carboxamide differ in electrostatic but not steric properties. Any effect of the mutations, if observed, would be due to electrostatic differences between the acid and its amide. Dependence of k_F on mutation was subject of our previous studies.[39,41,42] This study concerns independence of k_F of pH; mutations are discussed only so far as they help explain this finding.

As Table I shows, the k_F value for the Zncyt/pc(II) complex containing Asp42Asn is higher, and that for the complex containing Glu59Gln is lower, than the k_F value for the complex containing wild-type plastocyanin. Evidently, neutralization of charge by *amidation* of Asp42and of Glu59 in plastocyanin mutants does affect the rearrangement of the diprotein complex. For this reason, neutralization of charge by *protonation* of Asp42 and Glu59 in the wild-type plastocyanin is expected to raise and lower, respectively, the k_F values for Zncyt/pc(II). The plots in Figs. 3b and 3c are horizontal, showing no raising and lowering. The first explanation is refuted. Simultaneous deprotonation of Asp42 and Glu59 in the wild-type plastocyanin would both assist and hinder the rearrangement and produce little or no net change of k_F with pH. In this case, the single mutation Asp42Asn would abolish the former effect, while the single mutation Glu59Gln would abolish the latter. In other words, either single mutation would make the k_F sensitive to the deprotonation of the other acidic residue. Horizontal plots in Figs. 3b and 3c, however, rule out this scenario. The second explanation above is refuted, too. Evidence favors the third eplanation, namely that the acidic residues in plastocyanin within the complex remain deprotonated throughout the pH range.

To test this conclusion, we examined also the complex Zncyt/pc(II) containing the mutant Glu60Gln; see Fig. 3d. As Table I shows, the protein rearrangement is markedly impeded when Glu60 is neutralized by this amidation. Neutralization of Glu60 by protonation, in wild-type plastocyanin, likewise would impede the rearrangement. Absence of this effect, the horizontal plot in Fig. 3a, shows that protonation does not occur in the pH interval covered. Evidently, Glu60 remains deprotonated.

A closer look at rearrangement

There is much evidence for the general orientation of cytochrome *c* and high-plant plastocyanin in the electrostatically-stabilized binary comples: the basic patch around the exposed-heme edge in the former abuts the broad acidic patch in the latter.[48,58–63] During the rearrangement, the basic patch in cytochrome *c* moves from the acidic patch to an area near the upper edge of the acidic patch and between the acidic and hydrophobic patches, in the vicinity of Gln 88, in plastocyanin.[39,42]

Thorough NMR spectroscopic study of association between cytochrome *c* and plastocyanin could not show the protonation state of titratable residues in the protein complex because of fast H^+ exchange.[62] In the absence of direct evidence, we deduced this state of three residues in plastocyanin from kinetic evidence. Independence of k_F of pH shows that these three residues remain deprotonated during the rearrangement.

Previous research in this laboratory[41,64] showed that the rearrangement corresponding to k_F has low enthalpy of activation, possibly an indication that salt-bridges broken and

formed during the rearrangement compensate each other. This conjecture is supported by our present findings. The residues Asp42, Glu59, and Glu60 in wild-type plastocyanin remain deprotonated because they are engaged in salt-bridges.

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ИЗВОД

УТИЦАЈ pH НА КИНЕТИКУ СТРУКТУРНОГ ПРЕМЕШТАЊА КОЈЕ РЕГУЛИШЕ ПРЕНОС ЕЛЕКТРОНА ИЗМЕЂУ ЦИНК ЦИТОХРОМА *c* И ПЛАСТОЦИЈАНИНА. АНАЛИЗА ПРОТОНИСАНОСТИ АМИНОКИСЕЛИНСКИХ ОСТАТАКА У КОМПЛЕКСУ ДВА ПРОТЕИНА

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Пренос електрона од цинк цитохрома *c* до бакар(II)пластоцијанина у електростатички стабилизованом комплексу ова два протеина, Zncyt/pc(II), је регулисан структурним премештањем унутар овог комплекса [Crnogorac MM, Shen C, Young S, Hansson O, Kostić NM (1996) Biochemistry 35, 16465–74]. У овој студији истраживали смо премештање у четири различита комплекса Zncyt/pc(II), које цинк цитохром *c* гради са природном варијантом и трима једногубим мутантима пластоцијанина: Asp42Asn, Glu59Gln и Glu60Gln. Константа брзине реакције структурног премештања, k_F, се разликује за све ове четири врсте пластоцијанина и независна је од pH у интервалу од 5.4 то 9.0. Чињенице да је k_F зависно од мутације пластоцијанина а независно од pH указују да су аминокиселински остаци Asp52, Glu59 и Glu60 у природној варијанти пластоцианина депротовани (тј. анјони) у комплексу Zncyt/pc(II) у испитаном pH интервалу. Овај налаз сагласан је са замишљу да је раскидање соних мостова у почетној (и нереактивној) конфигурацији комплекса праћено успостављањем нових соних мостова у премештеној (и реактивној) кофигурацији комплекса.

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