Photolysis of the Histidine-Heme-CO Complex†

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Abstract: Photodissociation of the histidine-heme-CO complex has been investigated with picosecond to millisecond absorption difference spectra. The results suggest that both ligands of the heme group, histidine and carbon monoxide, are released after excitation. No independent phase for the dissociation of the histidine was observed, indicating that both axial ligands are rapidly dissociated. The recombination rates and kinetic difference spectra are consistent with the following scenario: After photodissociation the heme group recombines first with a water molecule and then with carbon monoxide; the final event is the replacement of the water molecule by histidine. The tetracoordinated heme has a Soret absorption peak at 432 nm and recombines with water with a rate of 2×10^4 M^{-1} s^{-1}.

Introduction

In hemoproteins, the heme is usually bonded by its iron atom to an imidazole nitrogen of a histidine (His) residue, which permits many important physiological functions such as the reversible binding of oxygen (in myoglobins and hemoglobins), catalysis of the reduction of hydrogen peroxide (catalases and peroxidases), electron transport (the cytochromes), oxidation of organic substrates (oxygenases and dioxygenases), and the reduction of dioxygen to water (the terminal oxidases). The interaction of heme with globin has been extensively studied, as well as the interactions with non-heme proteins such as serum albumin[1,2] and many model compounds.[3-5]

The role of the proximal histidine residue in hemoproteins is not yet fully understood. Although control of the ligand migration may be on the distal side, the restraint on the proximal side of the heme group also plays an important role in regulating the ligand affinity and the allosteric equilibrium in tetrameric hemoglobin (Hb). In fact when that stress exceeds a certain limit, as in Hb-NO with IHP at pH < 6.5, the iron histidine bond may be severed[6] and the liganded tetramer takes on the low affinity quaternary conformation. Similarly, in the Hb hybrids (α-nickel, β-iron), the histidine-nickel bond is severed, resulting in tetracoordinated nickel atoms; the tetramers are in the deoxy structure, with or without ligands on the iron atoms.[7,8] We report here the results of photodissociation experiments with the complex His-heme-CO, which also indicate a rupture of the histidine-iron bond.

Materials and Methods

Chlorohemin (Sigma Chemical Co., St. Louis, MO) was dissolved in 0.02 N NaOH just before the experiment. Samples were then diluted in 0.1 M sodium phosphate buffer at pH 7.2. Cosolvents ethanol or ethylene glycol (EGOH) were used to reduce dimerization or aggregation of hemes.[9] After equilibration of the solvents with 1 or 0.1 atm of CO, the ferric iron was reduced with sodium dithionite (Sigma) or dithiothreitol (Boerhinger Mannheim), when using ethanol as cosolvent. L-histidine (Sigma) was dissolved in water to give a stock solution of 0.5 M at pH 7.2, and then added to heme-CO solutions to obtain His-heme-CO. Samples were 10 or 100 μM in heme, as determined by the absorption spectra of the heme-CO species, with $\epsilon = 145$ mM^{-1} cm^{-1} at 406 nm in aqueous buffer, or $\epsilon = 175/mM^{-1}$ cm^{-1} at 410 nm in 80% (v/v) EGOH/buffer.

Static absorption measurements were made with a Kontron spectrophotometer (UV-930); the data were then analyzed on an IBM personal computer. EGOH (80% (v/v)) was used as solvent in order to measure the monomeric deoxyheme absorption spectrum.

Kinetic measurements were made after photodissociation with three different photolysis systems: (1) 100-fs laser pulses at 585 nm[10] for kinetics from 0.1 to 400 ps, (2) a 50-ps to 25-ns system using 30-ps pulses at 532 nm,[11] and (3) after photodissociation by 10-ns pulses at 532 nm (Quantel YG 585).[12] The different kinetic curves were normalized to the same absorption values in the overlap regions.

Results and Discussion

Equilibrium Binding of Histidine to H_2O-Heme-CO. The replacement of water by histidine in H_2O-heme-CO results in a large red shift of the absorption spectrum (Figure 1). In 80% EGOH the Soret peak absorption of H_2O-heme-CO is at 410 nm and that of His-heme-CO is at 418 nm. While the affinity of heme-CO for histidine is high (100 nM) in pure organic solvents,[13] a much lower affinity is observed for aqueous solutions likely due to competition with the water molecules:

$$\text{His} + H_2O\text{-heme-CO} \leftrightarrow \text{His-heme-CO} + H_2O \quad (1)$$

The observed equilibrium constant for the titration of H_2O-heme-CO with histidine (Figure 1) is 2.5 mM, in 80% EGOH/20% phosphate buffer at pH 7.2. There is little change between pH 7 and 12 for the histidine affinity or in the absorption spectrum. The affinity of free heme[13] or H_2O-heme (without CO) for histidine is much lower; in the same experimental conditions (80% EGOH) little change in the absorption spectrum was ob-

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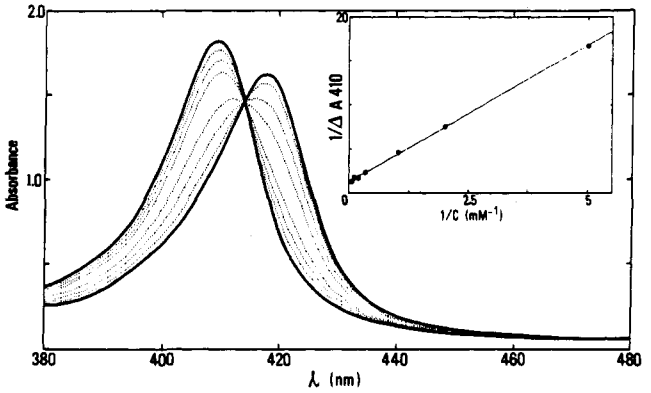

Figure 1. Titration of H_2O–heme–CO with histidine. Replacement of water by histidine causes a red shift in the Soret peak. From the titration curve (inset), the calculated affinity is 2.5 mM, in 80% (v/v) ethylene glycol/phosphate buffer at pH 7.2.

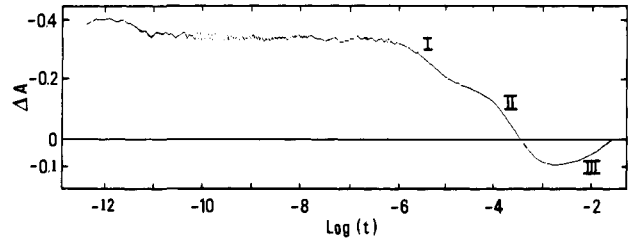

Figure 2. Kinetics after photolysis of His–heme–CO. After an initial relaxation process (5 ps), there is little ligand recombination between 10 ps and 100 ns. The three bimolecular phases are attributed to the binding of H_2O to heme (I) and CO to H_2O–heme (II) and histidine replacing the water in H_2O–heme–CO (III). Experimental conditions: observation at 436 nm, except for times less than 10 ps (at 438 nm); [heme] = 0.1 mM; 0.1 atm of CO; [histidine] = 10 mM; 100 mM phosphate buffer at pH 7.2 in 80% (v/v) EGOH.

served upon addition of histidine up to final concentrations of 30 mM.

Photochemical Properties of His–Heme–CO and H_2O–Heme–CO. The transient absorbance changes after photodissociation of His–heme–CO are shown in Figure 2. This composite curve was obtained by joining several kinetic traces, normalized in the overlap regions. The noise level therefore represents the particular detection system used. The detection wavelength was 436 nm, except for the first 10 ps (438 nm) in order to show more clearly the 5-ps relaxation process. There is little change in the signal between 10 ps and 100 ns at any detection wavelength, indicating little geminate recombination.

Unlike H_2O–heme–CO which shows a single exponential bimolecular phase for CO rebinding,[2] three phases are observed after photolysis of the His–heme–CO complex (Figure 2). The decay times for the three phases are of the order 1 μs, 100 μs, and 10 ms at 20 °C for samples in 80% EGOH equilibrated with 0.1 atm of CO.

Phase I. The rate of the first phase increases with higher percentage water, but is independent of the CO or histidine concentration. The time constant decreases from 10 to 1 μs for a change from 95 to 50% ethylene glycol. Since the recombination rate depends on the fraction of water, and was the same at pH 7.2 and 11, the reaction appears to reflect the binding of water (and not OH^-) to unliganded heme. The rate for this phase is similar for different cosolvents: At 20 °C, mixtures of water with ethanol, EGOH, or glycerol (same final percentage) gave the same rate (within a factor of 2), despite a change in viscosity of over 2 orders of magnitude. Results at lower temperatures showed a larger variation, with the more viscous solvents showing a larger dependence of the rate on the temperature.

Previous photolysis studies with dimethyl ester mesoheme in water-free benzene showed a 5-μs process reported to be the dissociation of the base;[13] for samples in aqueous solutions with cetyltrimethylammonium bromide (CTAB), the researchers still

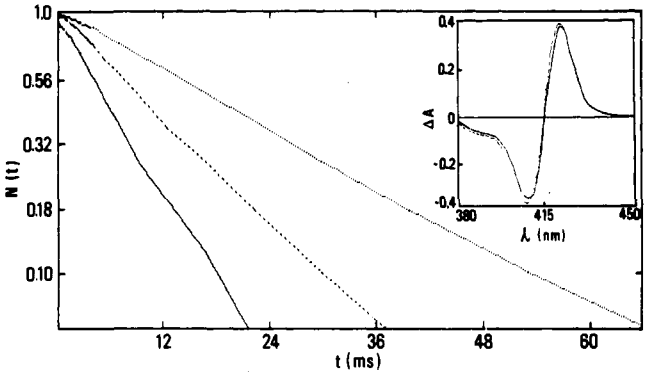

Figure 3. Final kinetic phase (III in Figure 2) after photolysis of His–heme–CO, at histidine concentrations of 10 (···), 20 (– – –), and 30 mM (—). $N(t)$ is the fraction of unreacted sites, after photolysis by 10-ns laser pulses at 532 nm; observation was at 410 nm. Samples were equilibrated with 1 atm of CO; other conditions are as for Figure 2. The inset shows that the kinetic difference (initial minus final) spectrum (—) for this phase is similar to the static difference spectrum (– – –) between H_2O–heme–CO and His–heme–CO.

claimed that the base remained bound to the heme after the CO molecule was photodissociated, although the signal for the base dissociation was noted as too rapid (<1 μs) to be observed.

The dependence of the rate of phase I on the percentage water indicates that this reaction could be water binding to tetracoordinated heme, or a replacement of the histidine by water.

Phase II. The rate of the second phase is proportional to the concentration of CO. The kinetic difference spectrum of phase II after photolysis of His–heme–CO was the same as the kinetic difference spectrum of H_2O–heme–CO and similar to the static difference spectrum between H_2O–heme–CO and H_2O–heme (measured in 80% ethylene glycol), indicating that CO rebinds to H_2O–heme in both cases. In other words, phase II for samples of His–heme–CO is identical to the single phase observed after photolysis of a sample prepared without histidine. Since the CO apparently rebinds to H_2O–heme, we conclude that, after phase I, water is bound to the heme.

Phase III. The rate of the third phase depends on the concentration of histidine. The kinetics of this phase at different histidine concentrations are shown in Figure 3. The insert shows that the kinetic difference spectrum of phase III is the same as the static difference spectrum of His–heme–CO minus H_2O–heme–CO, which confirmed the hypothesis that this component represents the replacement reaction described by eq 1.

The interpretation of phases II and III are clear, as the rate and spectral change correspond to known species which can be measured statically. In contrast, for the case of phase I, no static difference spectra could be obtained for tetracoordinated heme in aqueous solutions, to compare with the kinetic difference spectrum.

Transient Spectra of the His–Heme–CO Photoproducts. Loss of the histidine after photolysis is expected since the histidine has a much lower affinity for unliganded heme relative to heme–CO.[13,15] The question is on what time scale this occurs. In order to observe the dissociation of histidine and CO from the His–heme–CO complex, femtosecond photolysis studies were performed. The only submicrosecond event is a spectral relaxation (τ = 5 ps, Figure 2) which has previously been attributed to a relaxation from excited states; this phase (3–5 ps) is also observed in H_2O–heme–CO, myoglobin and hemoglobin with various ligands, and even systems without ligands such as deoxyHb.[14] The absorption changes were measured near the beginning and end of this phase: Figure 4 shows the difference spectra between the preflash complex His–heme–CO and the forms at 2 and 10 ps after photodissociation by 100-fs pulses. The transient spectrum for His–heme–CO at 1 or 100 ns after photodissociation (data not shown) are the same as that observed after 10 ps, with an isosbestic point at 426 nm.

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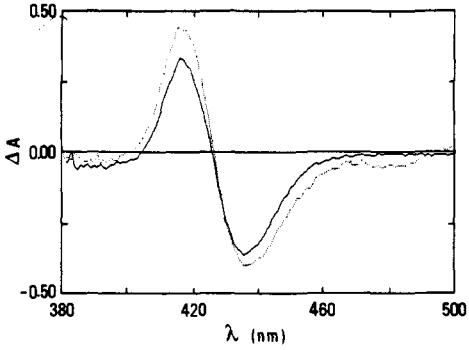

Figure 4. Kinetic difference spectra (initial preflash A minus $A(t)$ at t = 2 ps (---) and at 10 ps (—) after photolysis of His-heme-CO using 100-fs laser pulses at 585 nm. Solvent conditions are as for Figure 2.

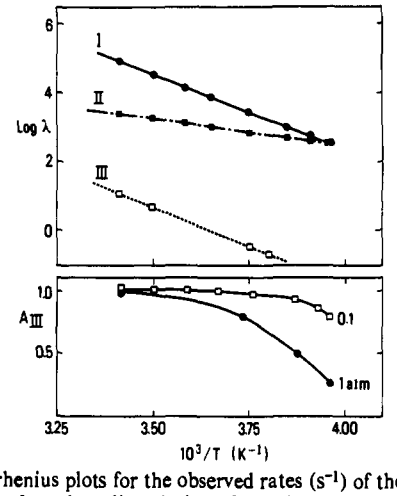

Figure 5. Arrhenius plots for the observed rates (s^{-1}) of the three phases (top section), after photodissociation of His-heme-CO in 96% EGOH equilibrated with 0.1 atm of CO, 10 mM histidine. The relative amplitude of phase III is shown in the lower section, at two different CO concentrations. When the CO rebinding phase (II) becomes comparable or exceeds phase I, the amplitude of phase III decreases.

Since no independent phase for the dissociation of the histidine was observed, there are two possibilities for the loss of the histidine: (1) immediately upon photodissociation of the CO molecule or (2) phase I represents a replacement of histidine by water. In the first scheme, tetracoordinated heme would be present from the picosecond to microsecond domain, whereas in the second case there would be no observable tetracoordinated intermediate.

Effect of Temperature. Additional measurements were made versus temperature to further characterize the transient states involved. For samples in 96% EGOH equilibrated with 0.1 atm of CO, phases I and III showed a similar temperature dependence with apparent enthalpies of 80 kJ/mol, while the rate for the CO recombination showed a smaller enthalpy of 34 kJ/mol. The Arrhenius plot (Figure 5) shows that the rates of phases I and II converge near −20 °C. The same sample was then equilibrated with 1 atm of CO to see if the CO rebinding phase (II) could precede the water binding phase (I). This does in fact occur and results in a decrease in the amplitude of phase III (Figure 5).

In the second scheme where phase I represents a replacement of histidine by water, the data showing the decrease in the amplitude of phase III versus temperature (Figure 5) can be easily explained: When the CO rebinds before phase I, the histidine is not lost and phase III decreases in amplitude.

Using the first scheme, where both ligands are immediately dissociated, the decrease in the amplitude of phase III can be explained if, after CO rebinds to free heme, the histidine rebinds immediately to the heme-CO complex:

$$\text{His-heme-CO} \rightarrow \text{heme} \rightarrow \text{heme-CO} \rightarrow \text{His-heme-CO}$$

The rate of histidine binding to heme-CO is in fact comparable to the rate of CO binding to heme, since the higher histidine concentration offsets the lower (by a factor of 5 for samples in benzene or 40 in CTAB solutions)[13] rate coefficient. Thus, the decrease in amplitude of phase III can be explained by either scheme, but places a lower limit on the rate coefficient for histidine binding to heme-CO with the first scheme.

Near 20 °C, the full factor of 10 increase in the rate of phase II was observed (1 versus 0.1 atm of CO). At −20 °C the CO rebinding rate was 7 times faster for the sample equilibrated with 1 atm of CO (where CO rebinding is the first event) than at 0.1 atm of CO (where phase I precedes the CO binding). Although the rates are not well determined when the kinetic phases are not resolved, the data indicate little change in the bimolecular rate for CO rebinding for the two cases, although the liganded form of the heme should be different.

In previous kinetic studies,[5,16,17] ligand recombination after flash photolysis of hexacoordinated heme complexes has displayed monophasic kinetics. Mesoheme dimethyl ester in aqueous suspension reacts with CO with a rate constant of 4×10^8 M^{-1} s^{-1}, while chelated hemes exhibit much lower rate constants, about 1×10^7 M^{-1} s^{-1}.[18] In the present experiments, when the CO binding phase (II) is slower than phase I, the same CO rebinding rate (2×10^8 M^{-1} s^{-1}) is observed after photodissociation of H_2O-heme-CO or His-heme-CO, similar to that for unchelated hemes. Since the rate and the transient spectrum for CO rebinding phase is the same for samples with or without histidine, we conclude that CO rebinds to H_2O-heme in both cases. However, after photolysis of His-heme-CO under conditions where the CO rebinding occurs before the water binding phase (at low temperatures for samples equilibrated with 1 atm of CO), the rate of CO recombination is only slightly decreased; this fact suggests that the histidine is dissociated before phase I.

Finally, we consider the dependence of the rate of phase I on the concentration of the different ligands. The scheme with photoproduction of tetracoordinated heme followed by the binding of water (as phase I) predicts a simple dependence of phase I on the water concentration, and no dependence on the CO or histidine concentration, as observed.

For the second scheme, where phase I is the replacement of histidine by water, the overall rate for phase I would be

$$R_I = k_{off}^{His} k_{on}^{H_2O} / (k_{on}^{H_2O} + k_{on}^{His}) \quad (2)$$

This rate coefficient would show a linear dependence on the percentage water, provided $k_{on}^{His} \ll k_{on}^{His}$ which reduces the form to

$$R_I = k_{off}^{His} k_{on}^{H_2O} / k_{on}^{His} \quad (3)$$

However, this implies a dependence of phase I on the histidine concentration as well, which is not observed. Samples in 80% EGOH equilibrated with 0.1 atm of CO were titrated with histidine from 4 to 28 mM. No change in the kinetics of phase I or II was observed.

This result is not consistent with a replacement reaction and favors the model where the two ligands are released upon excitation of His-heme-CO and leads to the following reaction scheme (Figure 6):

$$\text{His-heme-CO} \xrightarrow{h\nu} \text{heme} \xrightarrow{\text{I}} \text{H}_2\text{O-heme} \xrightarrow{\text{II}} \text{H}_2\text{O-heme-CO} \xrightarrow{\text{III}} \text{His-heme-CO}$$

The absorption spectra for the various species are shown in Figure 7; the spectrum for tetracoordinated heme (no. 2 in Figure 7) was calculated from the kinetic difference spectra (spectrum 1 plus the normalized absorbance difference at 200 ns). The same spectrum was obtained using the data at 10 ps or 1 ns, indicating that the three experimental systems were in agreement. The exact shape of the calculated spectrum depends on the normalization,

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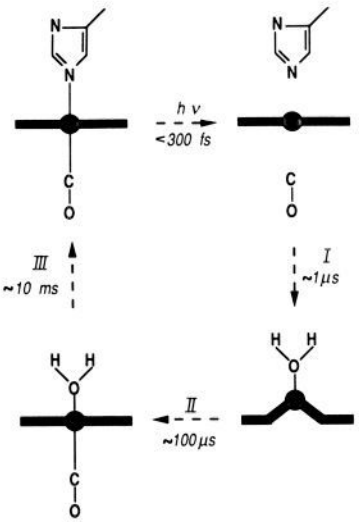

Figure 6. Schematic diagram of the reaction cycle after photolysis of His-heme-CO. The exact position of the iron atom relative to the heme plane is not known. After photodissociation of His-heme-CO, the tetracoordinated photoproduct reacts first with a water molecule and then with CO, and finally the water is replaced by the histidine.

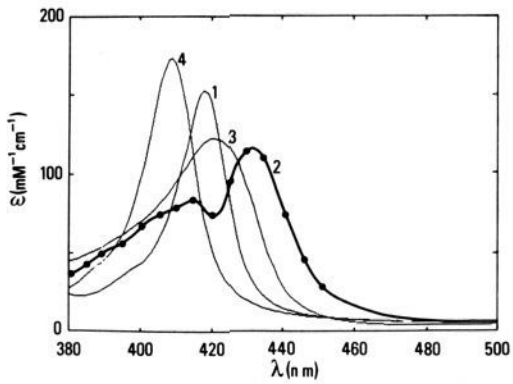

Figure 7. Absorption spectra in the Soret region of H_2O-heme-CO (4), His-heme-CO (1), and H_2O-heme (3) and the calculated absorption spectrum of the transient tetracoordinated heme after photodissociation of His-heme-CO (2).

but errors in this parameter could not produce this shape (with two peaks) from data of H_2O-heme-CO where photolysis produces a pentacoordinated species with a well-defined Soret band. Figure 7 shows that the Soret peak for tetracoordinated heme is further red shifted relative to that of H_2O-heme. The red shift and the shape of the Soret band are qualitatively similar to those reported for the unliganded dimethyl ester mesoheme in CTAB (Figure 2 in ref 18). A transient tetracoordinated spectrum has been reported for dimethylimidazole-protoheme-CO in CTAB solutions, which shows two peaks (380 and 429 nm) in the Soret region.[19]

The loss of both ligands could occur within the first few picoseconds, since no separate phase for dissociation of the histidine was observed. The spectral relaxation, of time constant 5 ps, is not interpreted as a signal for the loss of the histidine, since this phase also occurs for H_2O-heme-CO and nearly all hemoproteins studied. However, if this picosecond phase in other hemoproteins involves the histidine-iron bond, then it would still have to be considered as a candidate for representing the loss of the histidine. If both ligands are dissociated immediately, then phase I involves water binding to free heme; the second-order recombination rate constant for the water binding would be 2.4×10^4 M^{-1} s^{-1}.

The affinity of the heme for the histidine depends on the ligation state of the heme and the solvent. While the affinity of heme-CO for imidazole is high in benzene (10^8) and shows a phase for histidine dissociation 5 μs after photolysis of the CO, this affinity drops to 10^5 in aqueous solutions with CTAB and the dissociation phase was not observed.[13] In the present study, in aqueous solutions, the histidine affinity is even lower ($<10^3$); if the decrease in affinity is due mainly to an increase in the dissociation rate, such a phase would then be expected on the picosecond scale. This process would be accelerated during the picosecond jump in temperature just after photolysis; however, the actual dissociation rate during the first few picoseconds depends on the electronic configuration of the excited state.

In the study of chelated heme models in organic solvents, it is generally thought that only one ligand is released after the photolysis of hexacoordinated heme complexes. In chelated mesoheme a remarkable rate increase for CO binding has been reported as the pH is lowered. This was shown to be closely correlated with the pK_a of a spectral change,[18] which suggests the rupture of the iron-proximal base bond due to conversion of the base to its protonated form.

In deoxyMb at low pH, resonance Raman and optical absorption studies have also indicated cases where the imidazole-iron bond is broken.[20] A water or hydronium ion molecule was proposed to interact with the proximal or distal side of heme. It is deduced that the rupture of the imidazole-iron bond in deoxyMb at low pH is due to the protonation of the N_ϵ proximal histidine, but that does not occur in MbCO. Similar phenomena have been found in other hemoproteins.[21,22]

In HbNO at pH 6 in the presence of IHP, the imidazole-iron bond is broken or considerably weakened in the α chains, leading to a pentacoordinated heme complex. Thus, there is much evidence that the iron-histidine bond may be transiently or statically severed.[6] This might also occur in unstable hemoglobins where a water molecule could compete with the proximal histidine as a heme ligand.

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Registry No. H_2O-heme-CO, 64747-23-5; His-heme-CO, 136537-24-1; H_2O, 7732-18-5; His, 71-00-1; iron, 7439-89-6; heme, 14875-96-8.

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