Studies on Ligand Binding of Kidney Bean Leghemoglobin

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Absorption spectra of different ligand derivatives of kidney bean leghemoglobin *a* have been recorded.

The effect of pH on the absorption spectra of kidney bean leghemoglobin *a* has been studied. The *pK* of the acid-alkaline transition of the heme-linked water molecule is 8.25 and the *pK* for the acid dissociation of the heme group is 4.03.

Affinities of kidney bean leghemoglobin for two different types of ligands have been studied in comparison with soybean leghemoglobins *a* and *c* and sperm whale myoglobin. All these leghemoglobins have similar affinities for the small anionic ligand fluoride ion, and they are only slightly more accessible to this ligand than is sperm whale myoglobin. Differences in the primary structure or in conformation of these proteins are reflected in the affinity for the bulky ligand imidazole. The accessibility to imidazole increases in the order sperm whale myoglobin < soybean Lba < soybean Lbc < kidney bean Lba, and also low spin Lba < high spin Lba.

The results are discussed with respect to the amino acid sequences of the leghemoglobins.

Leghemoglobin, the monomeric oxygen-carrying hemoprotein of leguminous root nodules, belongs to the globin family of proteins. The amino acid sequences of four different leghemoglobins are known: soybean (*Glycine max*) Lba,^{1,2} kidney bean (*Phaseolus vulgaris*) Lba^{3,4}, broad bean (*Vicia faba*) Lba,⁵ and lupin (*Lupinus luteus*) Lba.⁶ Kidney bean Lba and soybean Lba differ at 35 of the 142 residues, and the primary structures of leghemoglobins differ as much as 80–90 % from the animal globin sequences, although the tertiary structures are closely related.⁷ A high oxygen affinity (see Refs. 8, 9) and a low isoelectric point (see Ref. 10) are considered to be characteristic of leghemoglobins but not of animal globins. The spectral properties of soybean leghemo-

globins *a* and *c*^{11–13} resemble those of animal globins, but spectral data of other leghemoglobins have been lacking. The aim of this study was to elucidate the correlation between the primary structure and molecular properties of different leghemoglobins.

MATERIALS AND METHODS

Leghemoglobins. Kidney beans (*Phaseolus vulgaris* var. Kaiser Wilhelm) were grown in a greenhouse at 26–32 °C in sea sand as described previously,^{10,12} using a 12 h day produced by artificial light. Leghemoglobin was extracted and purified as described previously¹⁰ but on DE-52 cellulose (Whatman, Maidstone, Kent.) instead of DEAE-Sephadex A-25. Soybeans (*Glycine max*) were grown in the conditions described above. The soybean leghemoglobin components *a* (high spin and low spin) and *c* were purified by the method used for the kidney bean leghemoglobin, but the chromatography was performed on a DE-52 cellulose column equilibrated with pH 5.6 sodium acetate buffer, and increasing acetate molarity from 0.01 to 0.1 at the same pH was used for elution.

Myoglobin. Lyophilized sperm whale myoglobin type II was from Sigma Chemical Co., St. Louis, Mo.

Heme protein concentrations were determined by the pyridine hemochrome method.²³

Absorption spectra were recorded in 1 cm quartz microcuvettes equipped with beam masks on a Cary 15 recording spectrophotometer thermostated at 20 °C. The sample volume was 200–300 μ l. A 1 mM stock solution of kidney bean leghemoglobin in water was diluted with appropriate buffer to about 50 μ M (700–450 nm) or to about 5 μ M (450–350 nm). The spectra of liganded leghemoglobin were recorded in sodium phosphate buffer, pH 6.0, μ 0.1, with the exceptions of the acetate complex in acetate buffer of the same pH and ionic strength and the alkaline form in sodium borate pH 10.1. Concentrated solutions of potas-

sium fluoride, potassium cyanide, sodium azide, nicotinic acid, and imidazole were brought to pH 6.0 with phosphoric acid. The ligand concentration in the cuvette was 0.87 M for fluoride, 8.5 mM for nicotinate and 0.20 M for the other ligands.

Acid-alkaline transition of kidney bean leghemoglobin was studied by recording the absorption spectra (750–350 nm) in the following buffers at an ionic strength of 0.1: sodium phosphate pH 6.00 and 7.00, Tris-HCl pH 7.98 and 8.55, and sodium borate from pH 9.18 to pH 10.59.

Heme group dissociation was determined from the spectral changes in the 450–350 nm region in sodium citrate buffers from pH 3.00 to pH 6.22. The spectrum of dissociated heme plus apoprotein was recorded in approximately 0.1 N HCl, pH 1.10.

Equilibrium constants for fluoride and imidazole dissociation were determined spectrophotometrically with a Beckman DU-2 in sodium phosphate buffer pH 6.0, μ 0.1 at room temperature (23 °C) using sperm whale myoglobin to check the method. Potassium fluoride binding was studied from spectral changes at 605 nm (leghemoglobins) or 607 nm (myoglobin) and imidazole binding at 533 nm (leghemoglobins) or 565 nm (myoglobin). Imidazole equilibria of the high and low spin forms of soybean Lba were studied similarly in phosphate buffers, μ 0.1, pH 5.9, 7.1 and 7.5. Titrations were carried out by successive additions of concentrated ligand solution. The absorbances were corrected for dilution, and the end point of each titration was checked by extrapolation of $[L] \rightarrow \infty$ in the graph of $\Delta A/[L]$ versus ΔA , where ΔA is the absorbance change produced by the added amount of the ligand and $[L]$ is the corresponding ligand concentration. All the pK values obtained had standard errors of ± 0.02 or less.

pH values were measured with a Radiometer pH meter 27 calibrated with Beckman pH 7.0 standard buffer, 0.05 M potassium biphthalate and 0.05 M sodium tetraborate.

Abbreviations. Hb, hemoglobin; Mb, myoglobin; Lb, leghemoglobin; CT, charge transfer bands. Lb²⁺ refers to leghemoglobin in the ferrous state and Lb³⁺ in the ferric state.

The term low spin is used for ferric hemo-proteins with one unpaired iron electron and high spin for those with five unpaired electrons.

RESULTS

Absorption spectra. The spectral properties of kidney bean Lba closely resemble those of hemoglobins and myoglobins in general (Table 1). Detailed comparison with the absorption spectra of soybean^{11–19} or lupin^{20–21} leghemoglobins is difficult because previously pub-

lished data are not expressed in numerical values. Spectral differences between kidney bean Lba and soybean leghemoglobins can be detected. For example, the oxy forms differ slightly in the wavelength of their Soret maxima and the ratio of the α and β band intensities. The Lb³⁺H₂O spectra also differ from each other. The Soret absorption maximum obtained here for soybean high spin Lba is at 403.5 nm ($\epsilon_{\text{MM}}=155$) and at 404.5 nm ($\epsilon_{\text{MM}}=142$) for both low spin Lba and kidney bean Lba, the soybean Lb-components having been purified by ion exchange chromatography, which does not give the pure low spin form. The ϵ_{MM} (400 nm) for soybean Lba²⁺F[–] was found to be 160 (in contrast to Ref. 18), so both soybean and kidney bean leghemoglobins have more intense Soret absorption for the fluoride complexes than for the Lb³⁺H₂O forms. This feature is not present in animal globins.

The major difference between the absorption spectra of the different leghemoglobins is the spin state of the ferric form below pH 7, where kidney bean leghemoglobin is much more in the low spin state than soybean or lupin leghemoglobins. Unlike kidney bean Lba,¹⁰ soybean Lba and Lbc can be resolved to pure high and low spin forms by isoelectric focusing in sucrose gradient, where the isoelectric points of the high and low spin forms differ by about 0.1 pH units.²⁴ Nicotinic acid has been shown to be the natural hemochrome ligand of soybean leghemoglobin,¹⁸ and it seems that kidney bean Lba is isolated mainly as the nicotinate complex.

Compared with the spectra of animal globins, some of the absorption maxima of leghemoglobins are shifted by a few nanometers towards lower wavelengths.

Acid-alkaline transition. The pK value of the acid-alkaline transition of kidney bean ferric leghemoglobin *a* was determined spectrophotometrically using eqn. (1).

$$pK = n\text{pH} + \log([Lb^{3+}OH^{-}]/[Lb^{3+}H_2O]) \quad (1)$$

where Lb³⁺OH[–] and Lb³⁺H₂O are the alkaline and acid forms, respectively, and *n* is the Hill constant, which gives the number of protons involved in the transition. The spectral changes at both 572 nm and 404 nm give a pK of 8.25, and the data are consistent with a single pro-

Table 1. Spectral values for kidney bean leghemoglobin α . The wavelengths (nm) of the absorption maxima or the shoulders (s) are given, and in parentheses are the corresponding millimolar absorption coefficients ($\text{cm}^{-1} \text{M}^{-1}$) based on heme concentration. The values for myoglobin and hemoglobin are from Ref. 25.

Ligand	Source ^a	Absorption bands				
		CT	α	β	CT	γ
Ferrous unliganded	PLba			556(13.4)		427(98.4)
	GLba ¹⁶			555(13.0)		427(103)
	s.w.Mb			556(11.8)		434(115)
O ₂	PLba		574(14.7)	539(14.4)		409(134)
	GLbc ¹²		574(15.1)	541(15.0)		411(139)
	s.w.Mb		581(13.3)	543(13.6)		418(128)
CO	PLba		563(13.8)	538(14.0)		417(210)
	s.w.Mb		579(12.2)	542(14.0)		423(187)
Ferrie H ₂ O	PLba (pH 6.0)	625(3.12)	566s(7.40)	532(10.1)	490s(9.10)	404.5(142)
	GLb (pH 5.2) ¹⁶	625(3.5)	560(5.0)	530(7.6)	496(8.5)	403.5(151)
	s.w.Mb (pH 6.0)	635(3.55)			505(9.47)	409.5(157)
OH ⁻	PLba (pH 10.1)		572(11.2)	539(12.2)		413(110)
	s.w.Mb (pH 11.5)		582(9.10)	542(9.48)		414(97.2)
N ₃ ⁻	PLba		573(9.97)	539(12.6)		414.5(123)
	Horse Mb		570(8.7)	540(11.2)		420(123)
CN ⁻	PLba			538(11.6)		417(130)
	s.w.Mb			540(10.7)		423(109.7)
Imidazole	PLba		562s(10.0)	533(12.8)		411(138)
	Horse Hb		560(12.5)	534(14.7)		411(105)
Nicotinate	PLba (pH 6.0)		557(11.0)	528(12.9)		407.5(121)
	GLb (pH 5.3) ¹⁶		557(10.1)	528(11.9)		407.5(119)
Acetate	PLba	622(5.95)	570(5.04)	532s(6.51)	494(9.08)	403.5(169)
	Horse Hb	620(5.5)			497(10.5)	404(178)
F ⁻	PLba	605(9.02)	576(7.00)	526(6.72)	485(9.56)	400(170)
	s.w.Mb	609(7.82)			490(8.31)	406(133)

^a Abbreviations: PLba, kidney bean (*Phaseolus*) leghemoglobin α ; GLba and GLbc; soybean (*Glycine*) leghemoglobin α , and β ; s.w.Mb, sperm whale myoglobin.

ton equilibrium, $n=1$ (Fig. 1). The pK values for the acid-alkaline transition of different hemoglobins and myoglobins all lie in a relatively narrow range,²⁵ and leghemoglobins are no exception to the general pattern. The pK values for soybean Lba and Lbc are 8.34 and 8.16, respectively,¹¹ whereas lupin leghemoglobin has a pK value of 8.7.²¹

No other heme-linked ionization could be demonstrated from the optical absorption spectra in the pH range 6.5–10, although the isosbestic points (at about 602, 525 and 479 nm) were not sharp enough to rule out this possibility. The slight instability of the absorption spectra above pH 10 indicated alkaline denaturation.

Dissociation of the heme group at low pH and ionizations below pH 7. The observed pK value for the cleavage of the prosthetic group from the protein is 4.03 and the stoichiometry of

the reaction is 1.0 (Fig. 1). The slightly higher acid stability of kidney bean Lba compared to soybean Lba¹⁴ may reflect minor differences in the environment of the heme group. Fig. 1 shows that subjecting kidney bean leghemoglobin to pH values below 5.5 causes dissociation of the heme group, and the pH range where the protein is spectrally stable is pH 6–10.

At pH 5–6.5 some ionizations occur in myoglobins and hemoglobins that may give rise to small changes in the spectrum of the acid form.²⁵ Soybean Lba of the low spin type²⁴ and lupin Lb²¹ have a spectrally distinguishable ionization in this pH region, whereas the absorption spectra of kidney bean leghemoglobin remain essentially unaffected by this pH change.

Equilibrium with fluoride. Fluoride is a small anionic ligand ($pK_{\text{HF}}=3.13$) that binds to the

Fig. 1. pH stability curves and the corresponding Hill plots for kidney bean leghemoglobin α . Dissociation of the heme group at acid pH is measured at 404 nm (●) and the acid-alkaline transition at 572 nm (○). Y is the fraction of undissociated and alkaline Lb, respectively.

sixth coordination position of the leghemoglobin iron, to form a purely high spin ligand complex.²⁶ Titrations of kidney bean leghemoglobin α as well as soybean leghemoglobins α and c with fluoride showed only minor differences in the affinities for fluoride of these proteins. The Hill plots of the formation of the fluoride complexes are shown in Fig. 2. Sperm whale myoglobin has the pK value 1.89, which agrees with the literature value.²⁷ Leghemoglobins have a slightly greater affinity for fluoride: kidney bean Lb α has a pK value of 2.04, and soybean Lb α and Lb c 1.95. An equilibrium constant obtained elsewhere for the soybean Lb α fluoride complex²⁸ differs markedly from the value obtained here. The present results show that no differences between the primary structures of leghemoglobins or even between leghemoglobins and myoglobin are reflected in the affinity for fluoride.

Equilibrium with imidazole. The reaction of ferric leghemoglobin with imidazole can be described by the following simplified equations:

Fig. 2. Hill plots of the binding of fluoride (filled symbols) and imidazole (open symbols) to different leghemoglobins: kidney bean Lb α (○), soybean leghemoglobin components α (Δ) and c (∇) and to sperm whale myoglobin (□). Y is the fractional saturation of the protein with the ligand.

$$K_{\text{app}} = [\text{Lb}^3 + \text{H}_2\text{O}][\text{Im}] / [\text{Lb}^2 + \text{Im}] \quad (3)$$

where K_{app} represents the observed dissociation constant for the reaction and the imidazole concentrations in eqn. (3) include all possible ionized species. At pH 6.0 the concentration of ferric leghemoglobin hydroxide is negligible compared to the concentration of acidic ferric leghemoglobin (1:200), but since the pK for imidazole dissociation is 6.98, the mixture contains 90.5 % of the positively charged imidazolium ion and 9.5 % of the uncharged imidazole. It has generally been assumed that the neutral form of imidazole reacts with the acidic form of the hemoprotein (*e.g.* Ref. 29), although it also has been postulated that imidazole reacts as if it were a charged ligand.³⁰

Imidazole has different affinities for the proteins tested. Sperm whale myoglobin has a

pK value of 1.22 for imidazole dissociation, which agrees well with the literature value.²⁰ Leghemoglobins from soybean have much greater affinity for imidazole than myoglobin, the corresponding pK values being 3.04 for Lba and 3.22 for Lbc. Kidney bean leghemoglobin is even more accessible to the ligand with a pK of 3.52. The corresponding association constants of sperm whale myoglobin, soybean Lba, soybean Lbc, and kidney bean Lba in the reaction with imidazole are in the ratio 1:66:100:200.

The slight difference between the imidazole equilibria of the high and low spin forms of soybean Lba is a result of the conformation differences, for the amino acid sequences are expected to be identical. The pK values for imidazole dissociation are 2.99 and 2.88 at pH 5.9, 3.85 and 3.73 at pH 7.1 and 3.98 and 3.98 at pH 7.5 for the high and low spin forms, respectively. At pH 7.5 the high and low spin forms of Lba have identical affinities for imidazole probably because of the dissociation of nicotinate from the low spin form.

Fig. 3. Hill plots of binding of imidazole to high spin (open symbols) and low spin (filled symbols) soybean leghemoglobin α at pH 5.9 (Δ), pH 7.1 ($\circ$) and pH 7.5 ($\square$).

DISCUSSION

The absorption spectra of porphyrins are shifted towards lower wavelengths when the electron-attracting power of the substituents on the porphyrin nucleus decreases.²¹ This kind of shift in the absorption spectra of ferric leghemoglobins, compared with myoglobins, may reflect weaker hydrophobic interactions between the heme and the globin. This would be consistent with the observation that at neutral pH the heme in ferric leghemoglobin is less tightly bound to the globin than in ferric myoglobin.²²

The tertiary structure of lupin leghemoglobin⁷ shows that the heme group is embedded in a hydrophobic cavity, like the heme in all other globins. The most serious hindrances to approaching bulky ligands such as imidazole are the practically invariable globin side chain groups of His E7, Val E11 and Phe CD1 (see Ref. 25). It seems that the residue E11 can be still bulkier without impairing ligand binding. Exceptionally E11 is leucine in kidney bean and soybean leghemoglobins and isoleucine in the invertebrate midge larva (with E7 Glu) and lamprey globins,²³ and all these globins have a high affinity for imidazole.^{24,25} Flexing of the helices and bending of the ligand permit binding even in these cases.

Residue B10 as well seems to be of interest in leghemoglobins. An invariable leucine is found in animal globins at this position,²³ which is occupied by an aromatic residue in all the leghemoglobins studied.¹⁻⁴ This residue is in heme contact at the ligand binding side²⁵ and it may well have an effect on the spectral and ligand binding properties of leghemoglobins.

Soybean Lbc is slightly more accessible to imidazole than soybean Lba, the ratio of the association constants being 100:66. The difference between nicotinate binding of these two proteins¹⁷ resembles the results obtained here. The amino acid sequence of soybean Lbc (Ellfolk and Sievers, in preparation) seems to differ in at least 9 amino acid residues from the soybean Lba sequence. In four of these replacements soybean Lbc is identical with kidney bean Lba, and in a further three the resemblance between soybean Lbc and kidney bean Lba is very close. Thus, soybean Lbc is more closely related to kidney bean Lba than

is soybean Lba, which is reflected in the affinities of these proteins for imidazole. It seems possible that some of the accessibility of the heme to bulky ligands may be attributed to the specific amino acid residues that are identical or closely related in kidney bean Lba and soybean Lbc, but dissimilar in soybean Lba and Lbc. The spin state of leghemoglobin also has an effect on the binding of a bulky ligand, as demonstrated by the slightly greater affinity for imidazole of the high spin form. This probably reflects greater flexibility of the high spin form.

Kidney bean leghemoglobin is mainly in the low spin state, but despite this it is more accessible to imidazole than the high spin form of soybean Lba. This difference must therefore be due to variations in the amino acid sequences, which are different at 35 positions of the total 142 residues. Most of these substitutions are conservative. Assuming that the leghemoglobins in question have the same three-dimensional structure as lupin leghemoglobin and myoglobin, an estimate can be made of the positions of the differences.

Interesting differences between soybean and kidney bean leghemoglobin structures seem to be at the following positions, respectively: A12 tryptophan or phenylalanine, E10 serine or lysine, H9 leucine or tryptophan, and the penultimate tyrosine instead of the termination of the protein chain. The residues in the E10 position in the vicinity of the ligand differ in size and charge, the first of which is probably not important if the residue projects into the solvent. Kidney bean leghemoglobin may have a greater affinity for positively charged ligands partly because the residue at E10 is neutral, whereas it has a positive charge in soybean leghemoglobin. The other amino acid residues mentioned above result in a slightly different distribution of aromatic residues in soybean and kidney bean leghemoglobins. Kidney bean leghemoglobin has more aromatic character than soybean Lba at the AB corner because of Trp A12, and also at the opposite side of the molecule because of the penultimate tyrosine. Soybean Lba, however, has more aromatic character in the middle of the H helix (Trp H9) close to the plane of the heme. These differences in the orientation of some aromatic

side chains could possibly affect the ligand binding of these two closely related proteins by changing the electronic structure of the heme.

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REFERENCES

1. Ellfolk, N. and Sievers, G. *Acta Chem. Scand.* 25 (1971) 3532.
2. Ellfolk, N. and Sievers, G. *Acta Chem. Scand. B* 28 (1974) 1245.
3. Lehtovaara, P. and Ellfolk, N. *FEBS Lett.* 43 (1974) 239.
4. Lehtovaara, P. and Ellfolk, N. *Eur. J. Biochem.* 54 (1975) 577.
5. Richardson, M., Dilworth, M. J. and Scawen, M. D. *FEBS Lett.* 51 (1975) 33.
6. Jegorov, C. A., Feigina, M. Yu., Kazakov, V. K., Chahparonov, M. I., Mitaleva, S. U. and Ovchinnikov, Yu. A. *Bioorg. Khimiya* 2 (1976) 125.
7. Vainshtein, B. K., Harutyunyan, E. H., Kuranova, I. P., Borisov, V. V., Sosfenov, N. I., Pavlovsky, A. G., Grebenko, A. I. and Konareva, N. V. *Nature (London)* 254 (1975) 163.
8. Wittenberg, J. B., Appleby, C. A. and Wittenberg, B. A. *J. Biol. Chem.* 247 (1972) 527.
9. Bergersen, F. J. and Turner, G. L. *J. Gen. Microbiol.* 91 (1975) 345.
10. Lehtovaara, P. and Ellfolk, N. *Acta Chem. Scand. B* 29 (1975) 56.
11. Ellfolk, N. *Acta Chem. Scand.* 15 (1961) 975.
12. Appleby, C. A. *Biochim. Biophys. Acta* 188 (1969) 222.
13. Appleby, C. A. *Biochim. Biophys. Acta* 189 (1969) 267.
14. Sievers, G. and Ellfolk, N. *Acta Chem. Scand.* 24 (1970) 439.
15. Imamura, T., Riggs, A. and Gibson, Q. H. *J. Biol. Chem.* 247 (1972) 521.
16. Appleby, C. A., Wittenberg, B. A. and Wittenberg, J. B. *Proc. Nat. Acad. Sci. U.S.A.* 70 (1973) 564.
17. Appleby, C. A., Nicola, N. A., Hurrell, J. G. R. and Leach, S. J. *Biochemistry* 14 (1975) 4444.
18. Ellfolk, N. and Sievers, G. *Biochim. Biophys. Acta* 405 (1975) 213.
19. Nicola, N. A., Minasian, E., Appleby, C. A. and Leach, S. J. *Biochemistry* 14 (1975) 5141.
20. Melik-Sarkisyan, S. S., Yarovenko, V. V., Shaposhnikov, G. L., Vladzhevskaya, L. P. and Kretovich, V. L. *Biokhimiya* 39 (1974) 711.

21. Atanasov, B. P. and Zhiznevskaya, G. Ya. *Mol. Biol.* 9 (1975) 491.
22. Ellfolk, N. *Acta Chem. Scand.* 14 (1960) 609.
23. Paul, K. G., Theorell, H. and Åkesson, Å. *Acta Chem. Scand.* 7 (1953) 1284.
24. Lehtovaara, P. *Finn. Chem. Lett.* Submitted for publication.
25. Antonini, E. and Brunori, M. *Hemoglobin and Myoglobin in their Reactions with Ligands*, North Holland Publ. Co., Amsterdam 1971.
26. Ehrenberg, A. and Ellfolk, N. *Acta Chem. Scand.* 17 (1963) S 343.
27. Bailey, J. E., Beetlestone, J. G. and Irvine, D. H. *J. Chem. Soc. A* (1968) 2778.
28. Wittenberg, B. A., Wittenberg, J. B. and Appleby, C. A. *J. Biol. Chem.* 248 (1973) 3178.
29. Diven, W. F., Goldsack, D. E. and Alberty, R. A. *J. Biol. Chem.* 240 (1965) 2437.
30. Beetlestone, J. G., Epega, A. A. and Irvine, D. H. *J. Chem. Soc. A* (1968) 1346.
31. Falk, J. E. *Porphyrins and Metalloporphyrins*, Am. Elsevier, New York 1964.
32. Ellfolk, N., Perttilä, U. and Sievers, G. *Acta Chem. Scand.* 27 (1973) 3601.
33. Dayhoff, M. O., Ed., *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, Maryland 1972, Vol. 5.
34. Mohr, P., Scheler, W., Schulmann, H. and Müller, K. *Eur. J. Biochem.* 3 (1967) 157.
35. Behlke, J. and Scheler, W. *Acta Biol. Med. Ger.* 21 (1968) 739.

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