R and T States of Fluoromethemoglobin Probed by Ultraviolet Resonance Raman Spectroscopy†

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ABSTRACT: Ultraviolet resonance Raman spectra are reported for the fluoride complex of methemoglobin (metHbF), with and without added inositol hexaphosphate (IHP), an allosteric effector known to stabilize the T quaternary structure. Environmental changes in Trp and Tyr residues give rise to the difference features, which are similar to those observed in the difference spectrum between deoxyHb and the CO adduct, consistent with T-state formation in metHbF in the presence of IHP. There are, however, important differences. The intensities of the difference signals are attenuated by about one-third, indicating a lower T-state population in the IHP-bound metHbF than in native deoxyHb. And a new signal is seen which arises from the interior tryptophans, probably reflecting a change in their H-bond status associated with the presence of fluoride as the sixth heme ligand in the T state. Implications of these results for the nature of the molecular forces opposing ligation in the T state are discussed.

The interplay between the structure of hemoglobin (Hb) and the mechanism of cooperativity has been a subject of extensive research (Perutz, 1979, 1990). The cooperativity is understood to result from a switch between states of low and high oxygen affinity, T and R, respectively, upon partial ligation of the four heme sites. One approach toward understanding the mechanism of cooperativity has been to study the structural changes in the ferric form of Hb, metHb, which are brought about by the binding of allosteric effectors such as diphosphoglycerate (DPG) and inositol hexaphosphate (IHP). Absorption and NMR studies (Perutz et al., 1974, 1978) have shown that the fluoride complex of metHb (metHbF) exists in the R quaternary state but is converted to the T form in the presence of IHP. The crystal structure of metHbF + IHP confirms that the subunit arrangement corresponds to the T state (Fermi & Perutz, 1977). A recent binding study indicates, however, that the conversion to the T state is incomplete in solution (Marden et al., 1991).

It is possible to probe the protein backbone and amino acid side chains via ultraviolet resonance Raman spectroscopy (UVRR) (Asher et al., 1983; Copeland & Spiro, 1985: Harada & Takeuchi, 1986; Hudson & Mayne, 1987; Su et al., 1989; Kitagawa, 1992). Excitation at wavelengths below 250 nm results in resonance enhancement of Raman active vibrational modes of aromatic side chains, with little or no interference from the heme group. We recently reported T – R difference RR features for both tyrosine (Tyr) and tryptophan (Trp) residues, of deoxyHb and its CO adduct, recorded simultaneously with 230-nm excitation (Rodgers et al., 1992). These RR markers of the R-to-T transition were found to arise from conformational changes at the $\alpha_1\beta_2$ and $\alpha_2\beta_1$ interfacial regions. The spectra specifically reflect structural perturbations at the interfacial residues Tyr α42 and Trp β37, whose hydrogen-bonding status is altered in the R-to-T transition.

In this report we present 230- and 219-nm excited UVRR spectra of metHbF with and without IHP. Comparison of these data with those of Hb clarifies the nature of the quaternary and tertiary contributions to the R-to-T transition.

EXPERIMENTAL PROCEDURES

HbA was prepared from fresh human blood by standard procedures (Antonini & Brunori, 1971). MetHb was prepared by oxidizing deoxyHb with 1.2 equiv of ferricyanide ion (Antonini & Brunori, 1971). The metHb was dialyzed overnight in 25 mM sodium phosphate buffer at pH 6.9 to remove the excess ferricyanide and the resultant ferrocyanide. After the preparation of deoxyHb, all steps were carried out in the dark to minimize the photoinduced formation of metHbCN in the presence of ferricyanide (Haldane, 1900). The metHb concentration was determined by converting a small aliquot to the cyanide adduct and measuring the absorption at 540 nm (Perutz et al., 1974). The metHb was then converted to the fluoro adduct by adding 1 M NaF to a final concentration of 0.1 M in NaF. The metHbF thus obtained was divided into two aliquots. Both aliquots were diluted to a final protein concentration of 0.25 mM with 25 mM phosphate buffer and one was made 5 mM in IHP. Na_2SeO_4 (0.1 M) or $NaClO_4$ (0.2 M) was added as internal standard. Song and Asher (1991) advocate the use of selenate as a nonperturbing internal standard and have reported that perchlorate perturbs the absorption and Soret-excited Raman spectra of metHbF. We have not observed these perturbations in the UVRR spectra of metHbF with twice crystallized $NaClO_4$ but have used selenate nevertheless. Integrity of the product was monitored via absorption spectra (Rots & Zandstra, 1984).

The UVRR spectrometer, using 230-nm excitation, has been described in the previous report from this laboratory (Rodgers et al., 1992). In order to generate 219-nm light, the 440-nm fundamental from coumarin 440 was frequency-doubled with a BBO crystal. Sample volumes of 0.5 mL were contained in a quartz NMR tube, which was spun around a stationary helical wire. A nitrogen atmosphere was maintained over the deoxyHb sample by delivering the gas into the NMR tube through a thin stainless steel tube. Spectral acquisitions were carried out in 1-h increments. To avoid buildup of photoreduced hemes (Kitagawa & Nagai, 1979), the metHb samples were discarded after 1-h exposure to the laser beam and replaced with fresh samples. The final spectrum was obtained by coadding 3 or 4 of the 1-h spectra. Before addition, each

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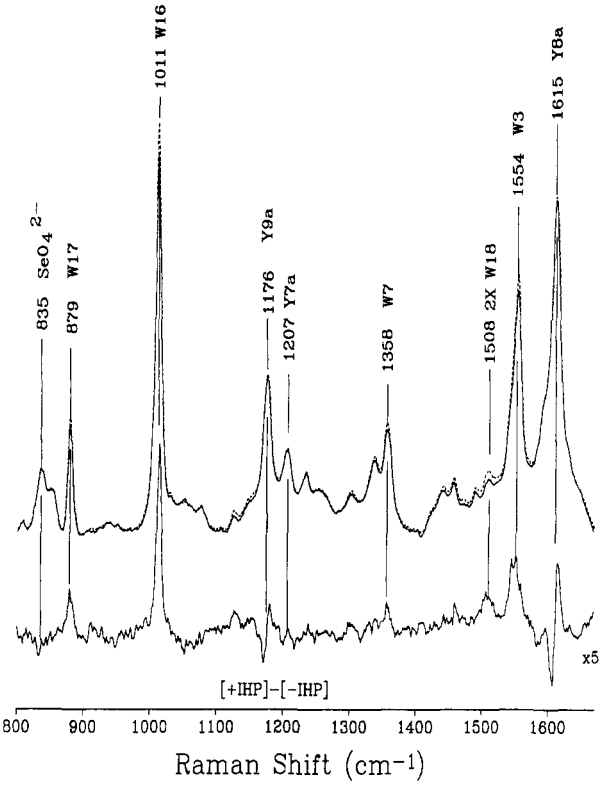

FIGURE 1: UVRR spectra (230 nm) of metHbF + IHP (dashed line) and metHbF without IHP (solid line) in the region 800–1650 cm^{-1}. The difference spectrum between the two is multiplied by a y-scale factor of 5.

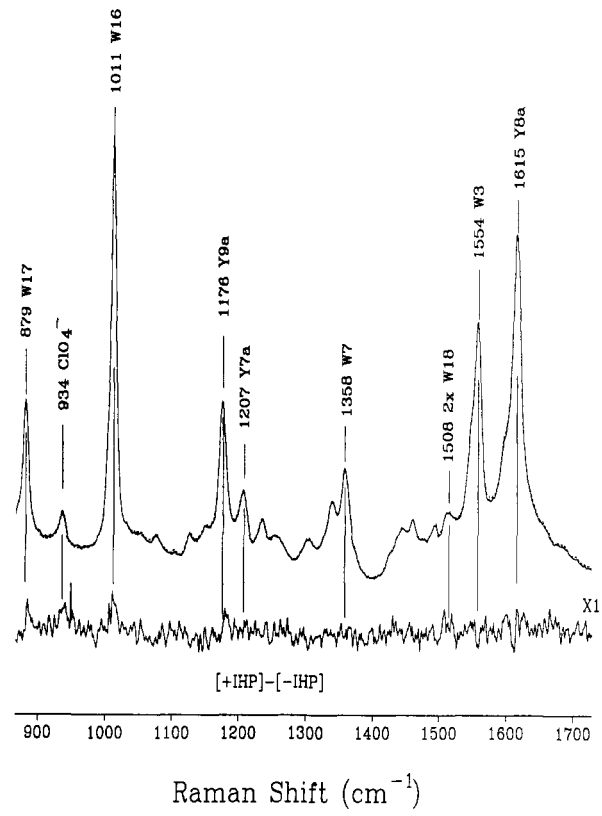

FIGURE 2: UVRR spectra (230 nm) of deoxyHb + IHP (dashed line) and deoxyHb without IHP (solid line) in the region 850–1700 cm^{-1}. The difference spectrum between the two is multiplied by a y-scale factor of 10.

spectrum was subtracted from the first spectrum taken for a given sample. If there were difference features the spectrum was discarded. The spectral intensities were normalized by adjusting the heights of the internal standard band to unity for all of the spectra. The normalized spectra were then subtracted to obtain the difference spectrum.

RESULTS AND DISCUSSION

T – R Difference Spectra Are Not the Same for Hb and MetHbF. Ultraviolet RR spectra of metHbF with and without IHP using 230-nm excitation are shown in Figure 1. The 835-cm^{-1} selenate peak was used to normalize the spectra for subtraction. Figure 3 compares the 230-nm excited difference spectrum for metHbF with and without IHP with the Hb minus HbCO difference spectrum. The latter has been interpreted as arising primarily from environmental changes affecting the aromatic residues at the $\alpha_1\beta_2$ interface, Trp β37 and Tyr α42 (Rodgers et al., 1992). The difference band at 1544 cm^{-1} is known to arise solely from an intensity change in the W3 band of Trp β37 because it is shifted in frequency from the position of the W3 band of the two interior Trp residues, α14 and β15 (1554 cm^{-1}). This is due to differences in the $\chi^{2,1}$ dihedral angle between the plane of the indole ring and that defined by the carbon atoms connecting it to the peptide backbone (Miura et al., 1989). As noted above, Trp β37 is in contact with different H-bond acceptors in the R and T states; it is also closer to the aromatic ring of Tyr α140 in the T state. The derivative-shaped difference signals at 1595 and 1615 cm^{-1} result from frequency upshifts of ca. 1.0 and 1.8 cm^{-1} in the tyrosine Y8b and Y8a bands, an effect that has been attributed to Tyr α42 acting as a H-bond acceptor from the protonated carboxylic acid group of Asp β99 in the T state (Rodgers et al., 1992). The remaining difference features are likewise thought to arise from the same environmental changes of the Trp β37 and Tyr α42 residues.

The metHbF + IHP minus metHbF difference spectrum bears a striking resemblance to the Hb – HbCO difference spectrum; all the features of the latter also appear in the former. There are, however, two important differences: (1) The metHbF spectrum exhibits a difference feature at the position of the interior Trp residue W3 bands (1554 cm^{-1}). It is, therefore, attributable to a tertiary interaction which is not present in the difference spectrum between Hb and HbCO. The possibility that this band is an artifact of IHP addition to the metHbF solution was excluded by a control experiment, in which the same concentration of IHP was added to deoxyHb (which is already in the T state). As shown in Figure 2, the IHP addition does not produce any detectable difference signals. (2) All the remaining features in the metHbF difference spectrum are attenuated by about one-third relative to those of the Hb T – R spectrum. The bottom trace in Figure 3 shows the result of subtracting the Hb minus HbCO difference spectrum, scaled by a factor of $^2/_3$, from the metHbF + IHP minus metHbF difference spectrum. Only the 1554-cm^{-1} difference band of the interior Trp residues remains in the trace.

UVRR Differences between Hb and MetHbF Are Limited to the T State. Figure 4 compares 230-nm excited UVRR spectra of metHbF and HbCO and of metHbF + IHP with deoxyHb. Within experimental error the metHbF and HbCO spectra are the same; the difference spectrum is featureless. This result indicates that the Tyr and Trp environments are insensitive to the nature of heme ligation, as long as the protein is in the R state. Although both proteins contain six-coordinate complexes, the heme is low-spin in HbCO and has short metal–ligand bonds, while it is high-spin in metHbF and has longer metal–ligand bonds which may prevent the Fe from assuming

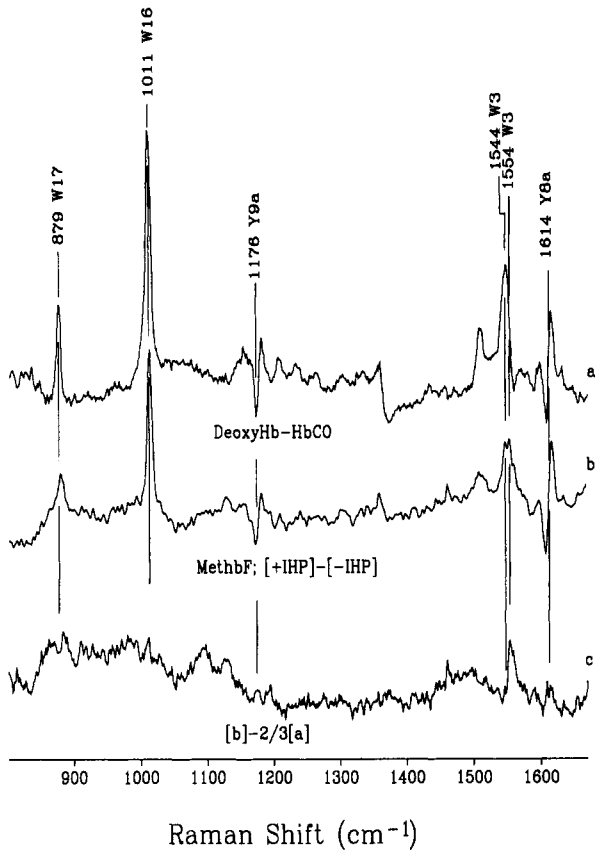

FIGURE 3: Comparison of the T – R state difference spectrum of Hb with that of metHbF. (a) DeoxyHb – HbCO UVRR difference spectrum using 230-nm excitation. (b) UVRR difference spectrum between the spectra of metHbF + IHP and metHbF. (c) Difference between spectrum b and spectrum a, after multiplication of the latter by a y-scale factor of $^2/_3$, to compensate for the incomplete conversion of metHbF to the T state by IHP.

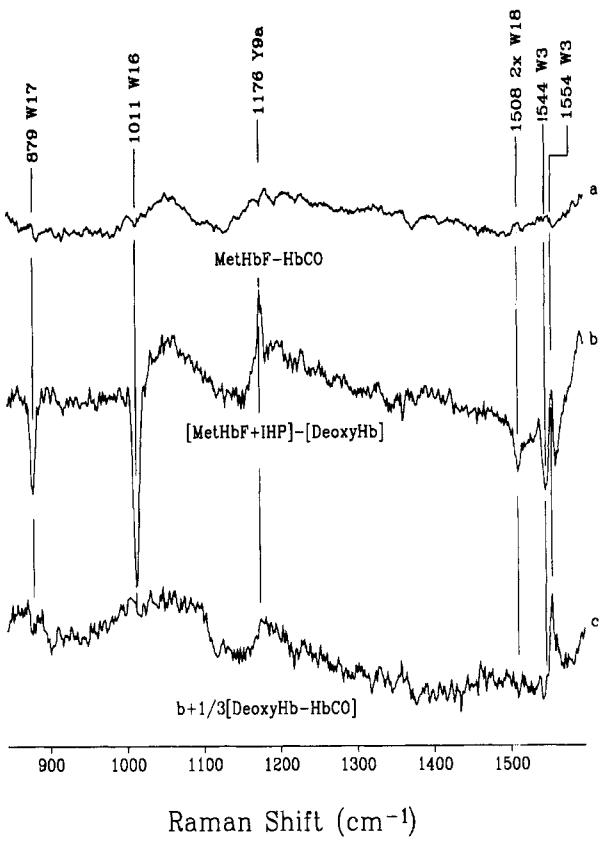

FIGURE 4: (a) MetHbF – HbCO difference UVRR spectrum. (b) UVRR difference spectrum between the spectra of metHbF + IHP and deoxyHb. (c) One-third of deoxyHb – HbCO spectrum added to spectrum b in order to remove the features due to the lower T-state population in metHbF + IHP relative to deoxyHb.

its natural R-state position in the heme plane (Deatherage et al., 1976). None of these differences alter the Tyr or Trp UVRR signals detectably.

There *are*, however, substantial differences between the UVRR spectra of metHbF + IHP and deoxyHb, accounting for the differences between the difference spectra (Figure 3). Even though both proteins are classified as being in the T state, on the basis of crystallographic and NMR evidence, the environments of the internal Trp residues are clearly not identical (Fermi & Perutz, 1977). The difference features between the UVRR spectra of metHbF + IHP and deoxyHb can be attributed to changes in the environment of internal Trp residues as well as incomplete conversion to the T state as is discussed in the next section.

Difference Spectral Intensities Establish Partial T-State Conversion in MetHbF in the Presence of IHP. The attenuation of the T – R difference features in the metHbF difference spectrum affects all the bands equally (with the exception of the new band at 1554 cm^{-1}) and indicates that conversion to the T state upon IHP addition is incomplete, even though IHP binding is saturated at the 5 mM concentration used in this study ($K_{diss} = 1.5 \times 10^{-3}$ mM) (Perutz et al., 1974). This diminution in the UVRR difference intensity is consistent with the recent analysis of CO binding to partially oxidized Hb by Marden et al. (1991), which led to the conclusion that saturation of metHbF adducts with IHP converts substantially less than 100% of the protein molecules to the T state, while even in the absence of effector molecules, a significant fraction of the high-spin metHbF is already in the T state. For metHbF, Marden et al. estimated the T-state content to be 29% in the absence of IHP and 72% in its presence. Since we subtract the spectrum of metHbF from that of metHbF + IHP to obtain our difference spectrum, the T – R signal strength should be 43% of the full T – R amplitude seen in the deoxyHb – HbCO. The actual factor is about $^2/_3$, suggesting that Marden et al. (1991) may have overestimated the T content of metHbF or underestimated the T content of metHbF + IHP, perhaps owing to limitations of the two-state model used in their analysis. Despite the quantitative discrepancy, there is gratifying agreement between these two very different experimental approaches that IHP binding to metHbF induces T-state formation only partially, previous assumptions to the contrary (Marden et al., 1991) notwithstanding. The crystal structure of T-state metHb obtained by oxidizing deoxyHb in an acrylamide matrix (Anderson, 1973) shows some Fourier difference features at the $\alpha\beta$ interface, relative to deoxyHb, giving an indication of the forces that lower the equilibrium T-state population in solution, even when the IHP binding site is fully occupied.

Interior Trp Monitors Tertiary Strain within the Ligated T State. The extra difference band at 1554 cm^{-1} in the metHbF + IHP minus metHbF spectrum or the metHbF + IHP minus deoxyHb spectrum (Figure 4c, where the equilibrium shift leading to lower T-state population has been corrected) reflects an environmental change of the interior Trp residues relative to *either* the R or the native T state. It is an effect specific to ligation in the T state. The interior Trp residues, α14 and β15, are located at nearly equivalent positions on the two A helices; the indole rings bridge across to the E helices that line the distal heme pockets, forming H-bonds to the OH groups of Thr α67 and Ser β72, respectively (Shaanan,

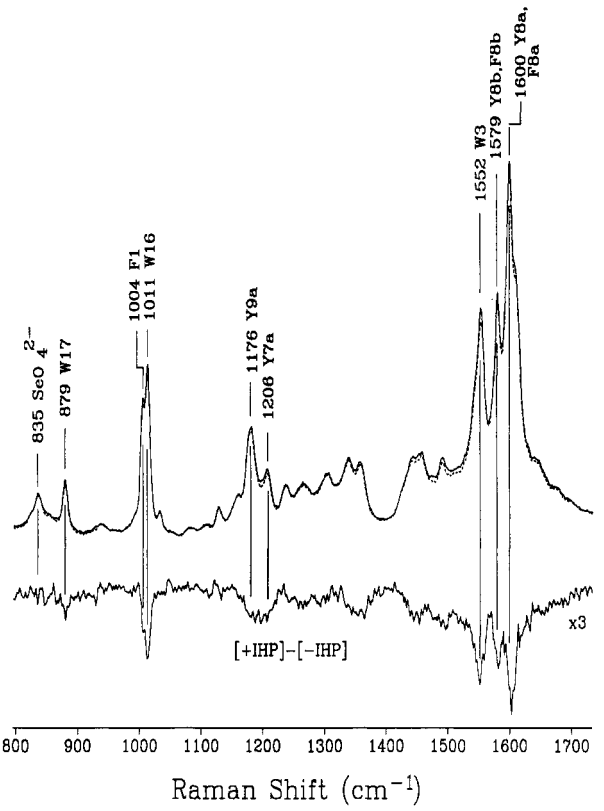

FIGURE 5: UVRR spectra (219 nm) of metHbF with IHP (dashed line) and without IHP (solid line) in the region 800–1650 cm^{-1}. The difference spectrum is multiplied by a y-scale factor of 5.

1983; Perutz et al., 1982). A possible explanation for the increased intensity of the 1554-cm^{-1} band is that the forces on the FG corner in the T state perturb the bridging H-bond interactions of the Trp α14 and β15 residues. This perturbation might, in addition, be mediated by H-bonds to the bound fluoride ions from the water molecules, which in turn are H-bonded to the distal His residues (Asher et al., 1981), which are part of the E helix near the Thr α67 and Ser β72 residues. The altered interactions of the internal Trp residues are an additional manifestation of the molecular strain that develops when ligated Hb is forced into the T state.

W17 Frequency Is Insensitive to H-Bond Changes between R and T States. The W17 band of tryptophan, found at ca. 880 cm^{-1}, has been reported to shift up by 5 cm^{-1} when the R state of metHbF is converted to the T state by addition of IHP (Kaminaka et al., 1990). This shift was claimed in transient UVRR spectra of the HbCO photoproduct and also when IHP was added to metHbF. In difference spectra obtained in this laboratory for Hb minus HbCO, however, W17 was observed to intensify with no frequency shift. Since the excitation wavelength was different in the two sets of experiments, 218 and 230 cm^{-1}, respectively, it could not be ruled out that the discrepancy might have arisen from selective enhancement of different Trp residues at the two wavelengths. The issue is important, since the W17 frequency has been reported, by Harada and co-workers, to be sensitive to H-bonding of the indole proton (Miura et al., 1989). The Trp β37 residue, at the critical $\alpha_1\beta_2$ interface, is H-bonded to different acceptors in the R and T states, the carbonyl group of Asn β102 and the carboxylate group of Asp α94, respectively, and might therefore be expected to exhibit a corresponding shift in its W17 frequency.

Accordingly, we recorded the UVRR spectra of metHbF, with and without IHP, using 219-nm excitation (Figure 5). The resulting difference spectrum shows a slight intensity loss but no frequency shift for W17. With 230-nm excitation (Figure 1), the intensity change is larger and opposite in sign, but there is no frequency shift. Moreover, the Hb – HbCO difference spectrum shows no W17 frequency shift when excited either at 230 or at 219 nm (not shown). Recent experiments by Kitagawa and co-workers (Kaminaka & Kitagawa, 1992) likewise indicate that W17 does not shift between deoxyHb and HbCO using 218-nm Raman excitation. Thus the change in the Trp β37 H-bond strength between the R and T states is insufficient to shift W17 detectably at either excitation wavelength.

The 219-nm excited spectra show several of the same Trp and Tyr bands seen in the 230-nm excited spectra, but also additional contributions from Phe residues and from amide vibrations. These additional and often overlapping contributions make detailed interpretation of the difference spectrum difficult. It is clear, however, that most of the difference bands are all negative, as Kaminaka et al. (1990) also observed, whereas at 230 nm, most of the difference bands are positive. This change in sign is attributable to a red shift of the excitation profiles of the aromatic residues in the T state, leading to higher intensities at the longer wavelength and lower intensities at the shorter wavelength. Model compound studies have revealed excitation profile red shifts associated with stronger H-bonding and/or a more hydrophobic environment (Rodgers et al., 1992).

CONCLUSIONS

The UVRR difference spectrum that developed when IHP was added to metHbF closely resembles that between deoxyHb and HbCO, but with diminished intensity. The R and T subunit interactions are essentially the same in metHbF as in native Hb, but the population of the T state is substantially less than 100% even when the IHP binding site is occupied. A new difference feature develops, which arises from the interior tryptophans and probably reflects a perturbation of their H-bonding due to interhelical forces generated by the presence of fluoride in the T state.

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