Time-resolved resonance Raman studies of carp hemoglobin

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1. INTRODUCTION

To understand the structural basis for the cooperativity of ligand binding to hemoglobin (Hb) it is necessary to know how the quaternary structure of the protein modulates the response of the binding site to the ligand and how the ligand brings about a switch in quaternary structure. To answer these questions it is essential to determine, as a function of quaternary structure, the functionally relevant structural changes that are generated by ligand binding. Time-resolved resonance Raman spectroscopy has been used to study the ligand-induced changes in the heme pocket of R and T states of human Hb's [1–3]. These studies [1,3] have also been effective in relating reactivity to structural features of the transient species of Hb which occur immediately after photodissociation. By focusing on the frequency of the iron–proximal histidine (F8) (Fe–His) stretching mode it is seen [2,3] that ligand binding causes a change in the heme–proximal histidine linkage that results in an increased frequency for the Fe–His stretching mode in the photolyzed species (at 10 ns) relative to that of the corresponding deoxy species. For both the deoxy [4,5] and the photolyzed [2,3] species the R state Hb's have the higher frequency. Here, we show that this same type of structural response, of the binding site to the ligand as well as the sensitivity of this response to quaternary structure is also in evidence in carp hemoglobin. The liganded derivatives of this hemoglobin can be reversibly stabilized in either the R or the T configuration [6]. In addition by comparing the yields of geminate recombination [7–9] we relate this same quaternary structure-sensitive degree of freedom to the kinetic constants that contribute to cooperativity.

2. METHODS AND MATERIALS

Hb (carp) was prepared as in [10] and stored as COHb. The oxy derivative was generated by photolyzing the COHb at 2°C under a constant flow of O_2. The resonantly enhanced Raman spectral line associated with the Fe–His stretching mode in the transient species was generated by using the 4350 Å (10 ns, ~1 mJ at 10 Hz) output of a dye laser (Lambda Physics 2002) pumped by an excimer laser (Lambda Physics 100). The O_2 geminate recombination studies required the use of the 4700 Å output (10 ns, $\ll$1 mJ at 10 Hz) of a nitrogen laser (Molection) pumped dye laser. The 4700 Å excitation was used in order to decrease the amount of repetitive photolysis to the point where a detectable signal from ligated material could be observed. In both experiments a single 10 ns pulse acted as the photodissociating pulse and the probe pulse. Consequently, the frequencies of the Fe–His stretching mode observed in the spectra are characteristic of the ligand-free transient species occurring within 10 ns of the photolysis. Similarly the relative yield of ligand free and ligand bound heme for the high and low affinity forms of Hb (carp) observed over the 10 ns pulse is a measure of the relative amount of geminate recombination that has occurred over this time scale for the 2 protein structures. The Raman signal was collected, dispersed, averaged and processed as in [1,2]. The temperature of the Hb solutions (~50–80 μm for 4350 Å, ~100 μm for 4700 Å) was maintained at

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4°C. The high and low affinity forms were stabilized in 0.1 M Tris (pH 8.9) and 0.1 M bis Tris (pH 5.7) + 20 mM IHP (inositol hexaphosphate), respectively.

3. RESULTS AND DISCUSSION

Fig.1 shows the portion of the resonance Raman spectra of several different forms of Hb that displays the frequency of Fe–His stretching mode. For the photolyzed species the spectra reflect the Fe–His stretching mode for the transient ligand free (deoxy) heme that occurs within 10 ns of photolysis. The frequency for photolyzed CO Hb A (human adult Hb, pH 8.9) is at ~232 cm^{-1} (fig.1) while frequencies at ~225, 217 and 215 cm^{-1} are seen for photolyzed CO Hb (carp) at pH 8.9, deoxy

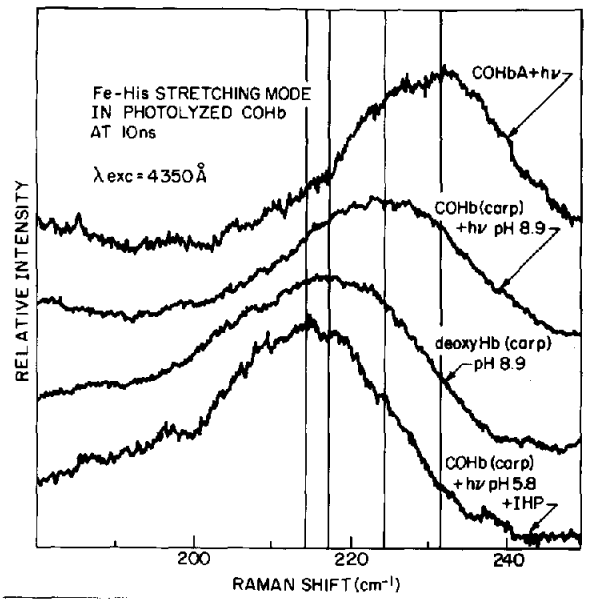

Fig.1. The response in Hb (carp) of the frequency of the iron-proximal histidine stretching mode to ligation as a function of pH and IHP. The spectrum of photolyzed COHbA at high pH is also shown. All the spectra are of deligated hemes; in the case of the photolyzed species the heme environmental is still that of the parent ligated species. Not shown is the spectrum of deoxy Hb (carp) at low pH which displays a band that is shifted 1–2 cm^{-1} to lower frequency relative to the corresponding photolyzed material whose spectrum is seen at the bottom of the figure. The frequencies observed for the deoxy species are identical to those in [11].

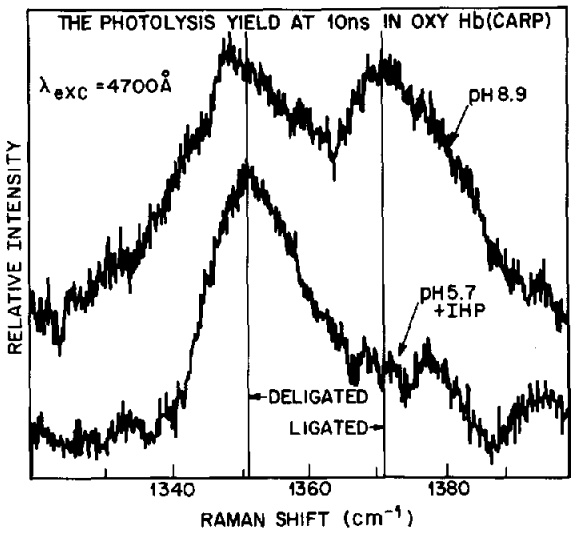

Fig.2. The effect of quaternary structure on the yield of photolysis at 10 ns for oxy carp hemoglobin. At high pH this Hb has the high affinity or *R*-like quaternary structure, whereas at low pH in the presence of IHP it has the low affinity or *T*-like structure.

Hb (carp) at pH 8.9 and photolyzed CO Hb (carp) at pH 5.7 + IHP, respectively. Not shown are the frequencies for deoxy Hb (carp) at pH 5.7 and photolyzed CO Hb (carp) pH 5.7 in the absence of IHP. These 2 species give rise to frequencies at 214 cm^{-1} and 225 cm^{-1}, respectively. The results for the corresponding photolyzed O_2 Hb were essentially the same.

In fig.2 is shown a portion of the high frequency Raman spectrum of Hb that is sensitive to the presence of ligand. The frequency of the displayed Raman peak (ν_4, band I, oxidation-state marker band) is very sensitive to whether or not the 6 ligand is bound to the heme. For ligand bound hemes (Fe^{2+}) the frequency is in the 1370–1380 cm^{-1} regime whereas for deoxy-heme (Fe^{2+}) the corresponding frequency is in the 1350–1355 cm^{-1} regime. We see the difference between the 2 forms of O_2 Hb (carp) in the relative yield of ligand-bound and ligand-free (deoxy) heme averaged over a 10 ns pulse that is both photolyzing and probing (fig.2). The spectra which are taken under identical conditions (sample concentration and pulse power) indicate that at 10 ns the high affinity form has substantially more ligand bound heme

than does the low affinity form (low pH + IHP). The results were qualitatively the same for samples under either air or 1 atm of O_2. Although the signal to noise is not high in these spectra because of the poor resonance conditions (4700 Å excitation) it is possible to detect for the peaks associated with ligand-free heme a shift to lower Raman frequency for the high *vs* low affinity forms.

In human deoxy-hemoglobins (normal, mutant and chemically modified) the Fe–His stretching mode shifts from ~ 215 cm^{-1} for the *T*-state species to $\sim 221 \pm 3$ cm^{-1} for the *R*-state species [4,5]. In this study as well as in [11] similar frequencies were observed for the low- and high-affinity forms of deoxy-Hb (carp). For the photolyzed human Hb's both the *T*- and *R*-states the frequency of the Fe–His stretching mode was increased in the 10 ns transient relative to the corresponding deoxy forms [2,3]. Thus, in going from deoxy-Hb's (*T*-state) to photolyzed *T*-state human Hb's [e.g., NOHbA + IHP at pH 6.8, COHb (Kansas) + IHP at pH 6.8] the frequency increases from 216 to ~ 222 cm^{-1} while the corresponding increase for the *R* state is from 221 ± 3 [e.g., deoxy NES des-Arg Hb at pH 8.9, Hb (Kempsey) at pH 8.9] to 232 cm^{-1} for photolyzed *R* [e.g., CO-HbA, NOHbA, O_2HbA, COHb (Kempsey) at pH 8.9]. At low pH in the presence of IHP, Hb (carp) is known [12] to bind O_2 non-cooperatively with very low affinity. Under these conditions the frequency of Fe–His stretching mode for both the deoxy (214 cm^{-1}) and the photolyzed species (215 cm^{-1}) is consistent with the protein having a *T*-like quaternary structure. At this pH but in the absence of IHP where this Hb retains cooperatively [12], the frequency of the Fe–His mode for the transient is much higher, having the same value (225 cm^{-1}) as the transient at higher pH (8,9) where the protein is locked into the *R*-like quaternary structure. Thus we have the following frequencies for the Hb (carp): deoxy-*T* 214 cm^{-1}; photolyzed *T* 215 cm^{-1}; deoxy-*R* 217 cm^{-1}; and photolyzed *R* 225 cm^{-1}. It follows that Hb (carp) exhibits a frequency behavior that is qualitatively similar to human Hb. Both the low- and high- affinity forms of Hb (carp) display an increase in frequency in going from the stable deoxy to the corresponding photolyzed product of either O_2 or CO forms. Although the frequencies for the photolyzed species are lower in Hb (carp) than the values for HbA the ordering is maintained with the high affinity form having the higher frequency for both for the deoxy- and photolyzed derivatives. A difference in the ligand-free hemes is also evident in the frequency of the Raman peak (ν_4) (fig.2). The frequency of this Raman band is also sensitive to quaternary structure for both of the stable deoxy forms [13] as well as the ligand-free transients derived from photolyzed ligand-bound forms [1]. This result is not surprising since it has been established that the frequencies of the Fe–His stretching mode and ν_4 for both deoxy [11] and photolyzed species [3] are related through an inverse linear correlation.

The spectrum of the transient reflects the influence upon the Fe–His linkage of the heme environment averaged over the 10 ns subsequent to photolysis. On this time scale, the heme has relaxed to an Fe^{2+} high-spin deoxy heme with the iron displaced from the plane of the porphyrin [2,3]. Large scale motions of protein that influence the heme environment are, however, not detectable until many 100's of ns later [3,14,15]. Consequently we have at 10 ns, a deoxy-heme surrounded by the heme environment that 10 ns earlier had been associated with the ligand-saturated porphyrin and is now, by virtue of the induced ligand dissociation, rendered unstable. Because in both cases the chromophore is a deoxy-heme it is possible to ascribe differences in the resonance Raman spectra of the deoxy and photolyzed species (at ≥ 10 ns) to differences in the influence of the heme environment upon the specific degree of freedom associated with the Raman band in question. From a recent analysis [2] of the variation in the frequency of the Fe–His stretching mode in deoxy and photolyzed Hb's and Mb it was concluded that the variation in frequency results to a large extent from protein and ligand induced variations in the angle of tilt of the histidine (F8) in its own plane with respect to the heme plane. For a fixed iron displacement, the greater the tilt the larger the repulsive forces between the imidazole carbon and the pyrrole nitrogen which results in a frequency decrease because of the weakening of the Fe–His bond. Within this first-order description, the tilt angle for Hb (carp) follows the same progression as seen for human Hb's: deoxy *T* > photolyzed *T* > deoxy *R* > photolyzed *R*.

Since the Raman frequencies generated from

the transient Hb's reflect the environmental milieu of the photolyzed ligand, it seems that the early time dynamics of the ligand might well be sensitive to the same structural parameters or features that modulate the Raman frequencies. One such process that is readily studied is geminate recombination [7–9] which is known to occur both on the sub-ns [16] and many 10's of ns [7–9] time scale for photodissociated O_2. Recent studies (in preparation) on oxy- and nitroxy-Hb's have revealed that for human Hb and turtle Hb the yield of geminate recombination occurring over a 10 ns pulse correlates with the frequency of both ν_4 and the Fe–His stretching mode which are themselves correlated. It was observed that in a comparison of any 2 transient Hb's (at 10 ns) the higher the frequency of the Fe–His stretching mode, and the lower that of ν_4, the greater the ratio of recombined sites to ligand free sites. This result appears to extend to the longer 50–100 ns recombination process observed for CO [1]. This relationship holds for oxy Hb (carp) as well (fig.2). The high affinity form has both the significantly higher frequency for the Fe–His stretching mode and the higher ratio of recombined to ligand free hemes.

The relationship between the frequency of the Fe–His stretching mode and the yield of geminate recombination should extend to the macroscopic dissociation rates as well. The reasons are as follows: The yield for the sub-ns geminate recombination is to a first approximation determined by the relative heights of 2 potential barriers; one controlling the recombination process and the other the diffusion of the ligand away from the bonding site. Based on transient absorption studies [9] it appears that the latter is not especially sensitive to changes in the protein structure. It follows that it is the recombination barrier that probably scales with the frequency of the Fe–His mode. An explanation for the basis of this relationship is suggested from theoretical studies [8] that indicate that there is an increase in repulsive energy between the histidine and the heme associated with an increasing tilt angle of the histidine. The increase in the repulsive forces makes it harder to bring the iron into the plane of the heme upon ligand binding which both increases the barrier height for binding and destabilizes the ligand bound state. Using pulse-probe time-resolved Raman, it has been shown (in preparation) that the frequency of the Fe–His stretching mode in photolyzed Hb relaxes to the corresponding deoxy-value on the time scale of 100's of ns to 100's of μs. Hence, it appears likely that the same 'frozen' environment of the ligand-bound heme that regulates the yield of geminate recombination is that which also controls the first steps associated with the spontaneous loss of a ligand such as O_2. The resulting scenario is that upon the spontaneous rupture of a ligand–heme bond, the iron rapidly moves out of plane but the cavity configuration associated with the ligand-bound heme remains relatively frozen for a much longer time. For an *R*-state Hb the more vertical orientation of the histidine makes recombination a more probable event than for *T*-state Hb's where the protein forces produce a tilted histidine with the associated higher barrier for reassociation. The off rate for the high affinity form of O_2 Hb (carp) is significantly lower than for the corresponding low affinity species [6].

This analysis in conjunction with Szabo's transition state treatment of Hb [9], when also extended to the deoxy forms can qualitatively account for the difference [10] between O_2 and CO with respect to the contribution of the on-rates to cooperativity for Hb (carp). Whereas for CO the on rate is strongly dependent on quaternary structure it is less so for O_2. In Szabo's treatment [9], CO, which binds more slowly than O_2 (presumably because of the difference in the distal-side interactions) has a transition state for the on step that resembles the ligand bound configuration while for fast reacting O_2 it resembles the deoxy configuration. Consequently for CO the difference in the on rate between the high and low affinity form should be determined by the configuration that gives rise to the corresponding early time photolyzed spectra. However, for O_2, the spectra of the deoxy forms are expected to reflect the relevant configuration that determines the above on rate potential barriers. Since for carp the change in frequency for the low affinity species of the Fe–His stretching mode in going from deoxy to the photolyzed is only ~ 1–2 cm^{-1} whereas for the high affinity forms it is ~ 7–8 cm^{-1}, the *R*–*T* difference in frequency for Fe–His mode is substantially greater for the photolyzed than for the deoxy forms (3 *vs* 10 cm^{-1}). Therefore, to the extent that these frequencies reflect configurations of the Fe–His linkage that contribute to the on-rate barrier height (by

regulating the energy of the transition state) it is expected and indeed observed [6] that the quaternary structure-induced differences in the on rate are greater for CO than for O_2. On the other hand, there is no difference between CO and O_2 in the free energy of cooperativity which is a thermodynamic parameter originating from the properties of the initial deoxy state and the final ligand-bound state. Here, we have a strong indication that the heme proximal environment is very nearly identical for O_2 and CO bound Hb. Thus the ligand-induced changes at the heme-proximal histidine interface that are seen in the transient Raman spectra may well be the structural basis of both ligand-specific dynamics and cooperativity in Hb.

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