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Modulation of reactivity and conformation within the T quaternary state of human hemoglobin: the combined use of mutagenesis and sol-gel encapsulation

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Abstract

A range of conformationally-distinct functional states within the T quaternary state of hemoglobin are accessed and probed using a combination of mutagenesis and sol-gel encapsulation that greatly slow or eliminate the T→R transition. Visible and UV resonance Raman spectroscopy are used to probe the proximal strain at the heme and the status of the $\alpha_1\beta_2$ interface respectively; whereas, CO geminate and bimolecular recombination traces in conjunction with MEM (maximum entropy method) analysis of kinetic populations are used to identify functionally distinct T state populations. The mutants used in this study are Hb(N β 102A) and the α 99– α 99 cross-linked derivative of Hb(W β 37E). The former mutant which binds oxygen noncooperatively with very low affinity is used to access low affinity liganded T state conformations whereas the latter mutant is used to access the high affinity end of the distribution of T state conformations. A pattern emerges within the T state in which ligand reactivity increases as both proximal strain and $\alpha_1\beta_2$ interface interactions are progressively lessened subsequent to ligand binding to the deoxy T state species. The ligation and effector dependent interplay between the heme environment and the stability of the Trp β 37 cluster in the hinge region of the $\alpha_1\beta_2$ interface appears to determine the distribution of liganded T state species generated upon ligand binding. A qualitative model is presented, suggesting that different T quaternary structures modulate the stability of different $\alpha\beta$ dimer conformations within the tetramer.

Keywords

Hemoglobin; sol-gel; Raman; kinetics; geminate recombination; protein dynamics; allostery

Introduction

Allosteric transitions represent an important mechanism for regulating protein reactivity. Hemoglobin has been and continues to be the prototype for studying the molecular basis of allostery. For hemoglobin as well as for other allosteric proteins, well characterized initial and final endpoint structures form the basis for most mechanistic models put forth to account for the observed allostery. The MWC two state model (1), used to explain many of the salient aspects of allostery within the hemoglobin tetramer, was based largely on the premise that there are oxygenation dependent changes in the equilibrium between two functionally distinct quaternary states: the low affinity T quaternary state and the high affinity R quaternary state (2–6). These states are correlated with the T and R quaternary

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structures derived primarily from X-ray crystallographic studies on two stable endpoint Hb species: deoxyHb and fully liganded Hb respectively.

A growing number of studies indicate that a detailed molecular understanding of allostery in Hb requires consideration of more than two affinity states. X-ray crystallographic measurements(7) and NMR solution phase studies (8) have shown that fully liganded hemoglobin manifests an ensemble – the R^e ensemble – of energetically similar, but spatially distinct quaternary structures. In contrast, several high resolution crystallographic studies have shown that fully deoxygenated hemoglobin has virtually the same T quaternary structure under high salt and low salt crystallization conditions (9–11). Such a structural observation might reasonably be construed to imply that the T state should have a narrow range of ligand binding properties; however, this is not the case. The ligand binding properties of the T state show considerable variability in response to the presence of effectors (12–19), partial ligation (20, 21), partial replacement of the four heme-irons with other metals (16, 18, 19, 22, 23) and mutations associated with the so-called hinge region of the $\alpha_1\beta_2$ interface (24–26). Such observations suggest that the T state is capable of manifesting considerably more conformational and functional plasticity than is implied by the crystallographic studies of deoxy HbA.

Thermodynamic studies (20, 21, 24, 27) have revealed both a third affinity state and a set of symmetry rules that determine the energy cost of oxygenation for partially liganded intermediates that together imply the existence of low and high affinity T states. Oxygen titration studies on sol-gel encapsulated hemoglobin have also revealed low and high affinity T state populations (28, 29). More recently, an X-ray crystallographic study of several hemoglobins (30) that follows the sequence of ligand binding induced changes occurring within the T state upon ligation, revealed direct evidence for a range of accessible conformations associated with the liganded T state species. Based on crystallographic parameters, populations were also divided into low affinity T (LT) and high affinity T (HT) populations. The present study directly complements the crystallographic study by probing both functional and conformational properties of LT and HT species in both solution and sol-gel.

The current study extends two earlier works: one that used solution phase and sol-gel encapsulated Fe-Zn hybrids of HbA to probe T state conformational plasticity (31) and the other that probed the conformational and kinetic properties of the low and high affinity T state forms of encapsulated HbA (32). In the present study, the scope of the T state landscape is more broadly explored. Two mutants of HbA are used to access, both in solution and in the sol-gel, a range of stable functional forms of the T state even when fully liganded. The Hb(N β 102A) mutant is used to access lower affinity range of the T state conformational distribution; whereas, α XLHb(W β 37E), the Lys α 99 diaspirin cross-linked form of the Hb(W β 37E) mutant, provides access to high affinity T state conformations. The conformational and functional properties of these mutant forms are compared with the range of T state properties observed for fully liganded encapsulated HbA and partially liganded Fe-Zn hybrid forms of HbA both in solution and in sol-gel. The Maximum Entropy Method (MEM) is used to group and compare kinetically distinct populations thus allowing for a direct comparison of accessible T states among the species derived from the above hemoglobins.

The Hb(N β 102A) mutant had been shown to bind oxygen with reduced cooperativity and very low oxygen affinity (26, 33). The replacement of the β 102 asparagine with alanine eliminates the Asp α 194-Asn β 2102 hydrogen bond, a crucial interaction contributing to the stability of the R quaternary state, without perturbing the key interactions associated with T state stability. It has been demonstrated both that the crystal structure of the deoxy derivative

of this mutant is identical to that of the deoxy HbA and that the oxygen titration for this crystal reveals an extremely low P50 (>100 mm) comparable to that associated with the binding of oxygen to the T state crystals of deoxyHbA(26). Most significant is the absence of significant functional heterogeneity between α and β chains within the Hb(N β 102A) tetramer (34). Furthermore, in the more recent findings (Kwiatkowski, Karasik and Noble, private communication), Hb(N β 102A), either in the presence of IHP or with a crosslinking ($\alpha\alpha$ - diaspirin) modification, binds oxygen non-cooperatively ($n=1$) and with an extreme low affinity (P50 > 100) that is comparable to what is observed in oxygenation studies on crystals of the deoxy T state form of both this mutant and HbA (26). The results indicate that: i) the mutant adopts the same deoxy T state as HbA both with respect to structure and functional properties and ii) under solution conditions, the mutant exhibits functional properties indicating that it remains in the T state subsequent to ligand binding. All indications are that this mutant is an excellent model for accessing ligand binding-induced low affinity T state conformations under conditions where contributions from the R state are minimal.

Mutations at the β 37 position have been shown to have a profound effect on the ligand binding properties of the T state tetramer that can be directly linked to a disruption of elements of the T state tertiary structure that are associated with quaternary constraint (24, 25, 35, 36). Quaternary constraint refers to the T state-derived decrease in heme reactivity towards ligand binding relative to the reactivity of the isolated $\alpha\beta$ dimers. Hb(W β 37E) shows the largest such effect among those mutants that have been systematically examined. As with the other members of this series, the X-ray crystallographic results show that the deoxy derivative adopts the standard deoxy T quaternary structure close to that observed for HbA but upon ligand binding accesses what has been termed a high affinity T state (HT) conformation (30). The results to date indicate that this mutant: i) upon ligand binding readily accesses the HT state and ii) binds oxygen with little or no cooperativity. This mutation also has the added effect of destabilizing the tetramer. In the present study, the diaspirin Lys α 99 cross-linked form of this mutant is used to eliminate contributions to the measurements from dimers (26, 34). Aside from eliminating the potential complication of dimers, the cross-linking has been shown both in this and earlier studies to have minimal impact on the unusual T state ligand binding properties of the mutant (26).

The utilization of these two mutants to explore features of the tertiary and/or quaternary structure landscape within the confines of the T state is further augmented by using sol-gel encapsulation protocols (37–40). Encapsulation allows for the generation of liganded derivatives of proteins that retain conformational memory of the initially encapsulated deoxy derivative and only slowly evolve towards the more stable conformational distribution associated with the solution phase liganded derivatives (41). The presently employed encapsulation protocol (31, 38, 39, 42) either minimizes or, more typically, eliminates the contribution of the R state when CO is added to the initially encapsulated T state deoxy derivative.

Resonance Raman spectroscopy is used to probe key conformational elements for the solution phase and sol-gel encapsulated mutant hemoglobins. Visible excitation (435.8 nm) by a 8 ns pulse is used to generate the Soret enhanced Raman spectra of both stable deoxy derivatives and the 8 ns deoxy-like photoproduct of CO saturated derivatives (43–45). These photoproduct spectra reflect the impact of the initial prephotodissociation conformation of the CO derivative on the resulting five coordinate high spin ferrous heme. The frequency of the iron-proximal histidine stretching mode (referred to as $\nu(\text{Fe-His})$ in the subsequent text) is a direct probe of quaternary constraint within the T state (43–50). This frequency correlates directly with the CO rebinding kinetics (31, 32, 43–45, 51, 52). UV excitation at 229 nm is used to generate Raman spectra resonantly enhanced by the absorption of

aromatic amino acid chromophores, especially tryptophan and tyrosine (53–56). The well-characterized 229 nm UV resonance Raman spectrum from HbA contains tyrosine and tryptophan bands that are highly sensitive to functionally and conformationally important domains within the $\alpha_1\beta_2$ interface (57–67).

The functional properties of hemoglobins are probed through the geminate (68–71) and bimolecular CO rebinding kinetics that occur subsequent to photodissociation of the CO derivative using a 8 ns excitation. The resulting kinetics are analyzed through the Maximum Entropy Method to help identify distinct kinetic populations based on distinct distributions of bimolecular rebinding rates in the pseudo first order limit. The results clearly reveal multiple discrete T state populations that are functionally and conformationally distinct. These different T state populations are apparent albeit with varying amplitudes, in both HbA (including the Fe-Zn hybrids) and the two mutants. The spectra and kinetics provide clear insight into the origin of the range of reactivity associated with T state hemoglobins and provide distinct markers for LT and HT populations. Most significantly, the results show that all of the above Hbs can access the same kinetically distinct high and low affinity liganded T state populations which also validates the use of the above two mutants as model systems suitable for probing the T state conformational landscape.

Methods

Materials

All materials, including inositol hexaphosphate (IHP) and tetramethylorthosilicate (TMOS) were commercially obtained at the highest purity available. The potent allosteric effector 2, [4-((3,5-dichlorophenyl)amino)carbonylamino]phenoxy]-2-methylpropanoic acid (L35) (72) was obtained as a gift from Dr. I. Lalezari. Human hemoglobin was purified as described previously (73). The cross-linked derivative of the Hb(β 102A) mutant was prepared from α dimers cross-linked between the α 99 lysine residues using the method of Chatterjee et al. (74) and Snyder et al. (75, 76) and of the mutant Hb(β W37E) as described by Kwiatkowski et al. (25).

Sol-Gel encapsulation

We employed a sol-gel encapsulation protocol that has been shown in previous studies to exhibit a high degree of ‘locking in’ of conformational structure (31, 38, 39). When referring to the samples we use a notation that indicates the history of the samples as shown in the scheme diagram below. Square brackets are used to indicate the species and conditions present during the sol-gel encapsulation and aging. Any changes to the samples after gelation and aging (e.g the addition of a ligand or a change in bathing buffer) appear outside and to the right of the square brackets with subsequent additions added on the right. All the buffers and solutions were deoxygenated prior to gelation. The deoxyHbA stock was generated by nitrogen purging and reduction with dithionite. Anaerobic conditions were maintained at all steps prior to CO addition. UV/Vis measurements were taken before and after gelation to verify the ligation and oxidation state of the samples. The bathing buffer was 50 mM BisTris Acetate and 25% glycerol in volume at pH 6.5. The final protein concentration in the sol-gel samples was ~0.5 mM in heme.

Experimental procedures

To improve ‘locking in’ i.e. the minimizing of relaxation of non-equilibrium populations trapped within the sol-gel, the samples were kept at $\sim 4^{\circ}\text{C}$. The “clock” is started by a rapid addition of CO to the sol-gel encapsulated samples. CO binding to the heme is monitored using UV/Vis spectra. Typically the Hb sample converts to the fully carbonmonoxy derivative within 5 minutes of addition of the CO saturated buffer. Then the samples are subjected to the battery of spectroscopic and kinetic measurements.

Visible resonance Raman spectroscopy

Visible resonance Raman (VRR) spectra were generated using an 8 ns pulsed laser at 435.8 nm (obtained using a hydrogen filled Raman cell to Raman shift 532 nm pulses of a Nd:YAG (Continuum, Santa Clara, CA)). A detailed description is available elsewhere (31, 38, 39, 77).

UV resonance Raman spectroscopy

UV enhanced resonance Raman spectra (UVRR) were generated using a continuous wave (CW) laser at 229 nm as described previously (31, 59, 60, 78).

CO rebinding kinetics

CO recombination measurements were carried out by following the transient absorption of the sample at 442 nm. Excitation with 8 ns pulses at 532 nm at 1 Hz from a Nd:YAG laser (Minilite, Continuum, Santa Clara, CA) was used for the CO photodissociation. A greatly attenuated CW laser at 442 nm was used as the probe. Detailed description of the apparatus is available elsewhere (31, 59, 60, 78–80).

Maximum Entropy Method (MEM)

Kinetic traces were deconvoluted using Maximum Entropy analysis as a means of identifying and grouping kinetic populations as a function of solution conditions, protein modification, time, and degree of ligation. This treatment calculates lifetime distributions without any *a priori* assumptions about the shape of the distribution and is therefore model independent. MEM is especially well suited for analysis of the complex multi-decade traces generated in the present study. The use of MEM to extract kinetic constants associated with ligand rebinding in a range of hemoproteins is well established (81–86). The MEM analysis was performed using a previously described algorithm (87, 88) that is now part of a commercially available package contained within the analysis module in Felix 3.2 software (Photon Technology International, Lawrenceville, NJ). Our results are consistent with the data obtained in the earlier applications. Kinetic data generated by photolysis were analyzed by setting a maximum number of anticipated lifetimes and the expected lifetime limits, and limiting the χ^2 value to 1. Distribution moments and related parameters were evaluated to determine the shape of the resulting distributions. Fits to the data were performed several

times, using different lifetime limits. In each case the autocorrelation was centered at zero (± 0.1), which is considered an acceptable fit. In addition the residuals were unstructured and randomly distributed about zero. From these statistical parameters changes in the distribution were estimated to be within 5–15%. The robustness of the analysis was further tested by increasing the χ^2 to > 1 and monitoring the extent to which the number of peaks and peak positions varied. All of the peaks discussed in the present treatment showed minimal variation with respect to center position and relative amplitude. The reported peaks represent Gaussian distributions of lifetimes. The center points are the mean values of the Gaussian distributions. The half-width of the peaks represent ± 1 standard deviation which is similar to those obtained in the previous studies. The data sets were tested further by fitting the original kinetic data with a set of exponential functions using these center points as the rates and the half widths as the pre-exponentials. The decay curves were successfully reproduced with this treatment. The MEM program was also tested using computer generated decay curves, which were successfully fit and reproduced by the program. The results of the MEM analysis are presented as the reciprocal of the rates in order to have the distribution displayed on the same axis as the kinetic traces. For the MEM populations associated with the bimolecular recombination, the amplitudes were normalized to unity and the fractional contribution of the each of populations calculated. The fractional distributions for the bimolecular phases are included in Table 2.

Results

HbN β 102A: visible resonance Raman spectra: solution phase results

Fig. 1 shows the low frequency region of the Soret enhanced resonance Raman spectra of both stable and transient ferrous five coordinate Hbs. The lowest frequency peak shown (between 214 and 230 cm^{-1}) corresponds to the conformation sensitive iron-proximal histidine stretching mode, $\nu(\text{Fe-His})$. The frequency of this mode is a direct reflection of proximal strain. Proximal strain is a contribution to the quaternary constraint that causes reduced ligand affinity in the T state. Proximal strain originates from the added T state energy penalty associated with moving the iron into the plane of the heme macrocycle upon ligand binding (48).

For vertebrate hemoglobins, low frequencies for $\nu(\text{Fe-His})$ are indicative of proximal strain whereas high frequencies near or at 230 cm^{-1} are reflective of proximal enhancement. Proximal enhancement which is typically associated with the photoproduct of liganded hemoglobins arises from a compression of the iron-histidine bond due to the histidine being constrained by the protein to be close to the heme (47, 58). As a result there is a reduction in the energy cost associated with moving the iron in plane upon ligand binding.

The heme γ_7 mode band at approximately 300 cm^{-1} has also been shown to be sensitive to proximal strain with lower frequencies being indicative of increased proximal strain (89). The propionate sensitive band at 340 cm^{-1} is prominent and distinct for stable ferrous five coordinate hemoglobins whereas for the early time photoproduct it is reduced in intensity and poorly defined as a discrete band especially for the R state photoproduct (45).

Spectra a and b are a comparison between the deoxy derivatives of HbA and Hb(N β 102A)+IHP respectively. It can be seen that the two spectra are essentially identical, resembling previously described spectra from deoxy HbA. This result is consistent with the deoxy derivative of the mutant adopting a tertiary/quaternary conformation distribution that is very similar to that of deoxy HbA.

Spectra c through e are derived from the ferrous five coordinate heme generated within 8 ns of photodissociating the parent COHb. As such, the species yielding the spectra are typically

transient ferrous five coordinate heme species that still retain the influence of the unrelaxed (or nearly unrelaxed) liganded protein conformation (44, 45). It can be seen that the frequency of $\nu(\text{Fe-His})$ undergoes an increase in going from the stable deoxy forms to the photoproducts. The frequency of $\nu(\text{Fe-His})$ for the photoproduct of $\text{COHb}(\text{N}\beta 102\text{A})$ is lowest at 221.5 cm^{-1} in the presence of both IHP and L35 (not shown, see Table 1) and increases to 222.5 cm^{-1} with the removal of any one of the two effectors (only the +IHP is shown in the figure, see Spectrum c). In the absence of effectors, the $\text{HbN}\beta 102\text{A}$ photoproduct yields a still higher frequency at 226.3 cm^{-1} (see Table 1). In contrast the photoproduct frequencies from COHbA under similar conditions with and without IHP are 228 and 230 cm^{-1} respectively (Spectra d and e). It can be seen that the heme γ_7 mode band follows a similar progression although over a much narrower frequency range (300 to 304 cm^{-1}).

HbN β 102A: visible resonance Raman spectra from sol-gel encapsulated samples

Fig. 2 shows the same low frequency region of the Raman spectrum as in Fig. 1 but in this case the samples are encapsulated in a porous sol-gel matrix bathed in buffer. Spectrum a, derived from sol-gel encapsulated deoxy HbA is virtually identical to the solution phase spectrum shown in Fig. 1a. Spectrum b of encapsulated deoxyHb(N β 102A)+IHP, designated as [deoxyHb(N β 102A)+IHP] is seen to be nearly identical to that of the corresponding deoxyHbA sample (spectrum a). These results indicate that at least with respect to the quaternary/tertiary structure-sensitive proximal heme pocket environment of the deoxy derivatives of both HbA and Hb(N β 102A), the sol-gel encapsulation protocol does not noticeably perturb the solution phase conformations.

Spectrum c is from the 8 ns photoproduct of sol-gel encapsulated sample generated within an hour of the addition a large excess of CO saturated, IHP containing, buffer to [deoxy(HbN β 102A)+IHP]. The visible absorption spectrum of the sample indicated that the sample converted to the CO derivative within several minutes of adding the CO saturated buffer. Spectrum c illustrates that there is a small but relatively rapid change in conformation upon addition of CO that results in a slight decrease in proximal strain as reflected in the small frequency increase in $\nu(\text{Fe-His})$ and γ_7 . Spectra d and e are associated with samples in which the CO derivatives were directly encapsulated, i.e. [$\text{COHb}(\text{N}\beta 102\text{A})$ +IHP] and [COHbA] respectively. A comparison among spectra b, c and d shows that encapsulation greatly slows the CO binding induced transition from the equilibrium deoxy derivative of the mutant to that of the liganded derivative. It takes in excess of a month for the encapsulated deoxy+CO sample to achieve the end point spectrum as can be seen in the bottom of Table 1. In contrast the corresponding deoxy turned CO encapsulated HbA sample achieves the $222\text{--}223\text{ cm}^{-1}$ value within hours of the addition of CO. Table 1 contains the frequencies of $\nu(\text{Fe-His})$ for these samples as well as related samples including Hb(W β 37E) whose spectra are not shown.

UVR R spectra: overview

Whereas the visible resonance Raman results address conformational issues associated with the heme and the heme environment, the UV resonance Raman provides details associated with the T state constraints within the $\alpha_1\beta_2$ interface. Fig. 3 shows only the high frequency region ($1500\text{--}1660\text{ cm}^{-1}$) of the 229 nm excited UV resonance Raman (UVR R) spectrum ($800\text{--}1680\text{ cm}^{-1}$). This high frequency region contains bands that have been well-characterized with respect to specific conformational degrees of freedom associated with the hinge and switch regions of the $\alpha_1\beta_2$ interface (57, 61, 67, 90–93).

Fig. 3 shows the 229 nm excited resonance Raman spectra of deoxy (solid line) and CO (dashed line) derivatives and their difference spectrum (deoxy-CO) for: solution phase HbA

(series a); solution phase HbN β 102A+IHP (series b); [deoxyHbN(β 102A)+IHP] and [COHb(N β 102A)+IHP] (series c); and [deoxyHb(N β 102A)+IHP] and [deoxyHb(N β 102A)+IHP]+CO (series d). Note that the W3 band and the Y8a band are independently normalized with respect to their peak intensities in order to better expose the shift in Y8a and the intensity change in the Trp β 37-associated shoulder of the W3 band. It can be seen that the deoxy T versus liganded R differences seen for HbA in Series a are also in evidence for Hb(N β 102A) but with smaller amplitudes for the latter. For both solution and sol-gel samples, the deoxy versus CO derivative of the mutant (Series b and c) show a reduced shift in Y8a and reduced intensity changes both in the β 37 shoulder of W3 and in the β 37 sensitive 1511 cm^{-1} band. The deoxy versus liganded differences are nearly but not completely eliminated for the mutant in Series d where the encapsulated deoxy form is compared to the CO derivative generated from an initially encapsulated deoxy sample.

For Series a, the difference spectrum reflects the difference between the deoxy T state and fully liganded R state of HbA. In contrast, all of the subsequent Hb(N β 102A) difference spectra represent comparisons between the deoxy T state and either the equilibrium population of the liganded T state (Series b and c) or T state populations having tertiary conformations that are intermediate between the stable forms of deoxy T and liganded T (Series d). It is clear from these series of difference spectra that the $\alpha_1\beta_2$ interface does not function as a simple two state switch. Based on the established assignments for the features in the difference spectra, the UVRR data show that upon ligand binding within the T quaternary state there is a progressive loosening of the T state constraints within the $\alpha_1\beta_2$ interface. Similar ligand binding-induced loosening of the T state constraints were previously noted for Fe-Zn hybrids of HbA (31) and [deoxyHbA]+CO (32).

Geminate and Bimolecular Recombination

The functional properties of the different T state populations that were identified using Raman are now examined through CO recombination kinetics subsequent to photodissociation. Whereas combination rate measurements using mixing technology probe primarily the initial deoxy Hb population, the recombination following rapid photodissociation probes the initial population of the liganded derivative as well as populations that have undergone relaxation during the time course of the measurement. The initial geminate recombination phase most directly correlates with the population retaining the conformational distribution of the initial liganded or photoproduct species. The slower bimolecular or solvent phase recombination can reflect populations that have undergone relaxation subsequent to the initial photodissociation. The use of sol-gel encapsulation minimizes the relaxation effects due to the damping of both tertiary and quaternary state relaxation on the time scale of kinetic measurements.

Fig. 4 shows the range of kinetics observed for the rebinding of CO subsequent to photodissociation by an 8 ns pulse for solution phase samples at pH 6.5. Traces a and b, from COHb(α Zn/ β Fe) +IHP and COHb(N β 102A)+IHP+L35, respectively, show variation in the kinetic pattern for T state species. The initial rebinding occurring throughout the nanosecond to early microsecond time regime has been identified as geminate recombination (68, 69, 71). The two recombination phases observed for COHbA seen in trace c, occurring between 100 μsec and 1 sec, correspond to the bimolecular rebinding of CO molecules from the solvent (94). The fast and slow bimolecular phases correspond to R and T state combination rates respectively. Trace d is also derived from COHbA but under conditions of low photolysis which greatly reduces the conversion of photoproduct to T state population. As a consequence, there is no T state bimolecular rebinding phase in the kinetic trace and only the R state bimolecular phase is seen. All subsequent traces were obtained under essentially the same low photolysis excitation limit. The rebinding kinetics from COHb(N β 102A)+IHP+L35 shown in trace b reveal a marked decrease in the geminate

yield, an absence of the R state bimolecular phase, a sizable population displaying the T state bimolecular phase and what appears to be an intermediate phase occurring between the T state and R state bimolecular phases seen in trace c. Trace a, which was previously discussed in an earlier work (31), is derived from a species that has been designated as an extreme T state. It shows a geminate yield of near zero and no indication of an intermediate T state kinetic phase. The reduced geminate yields and T state bimolecular phases seen in traces a and b are consistent with previously reported patterns derived from T state CO derivatives of iron-metal hybrids of HbA (31, 95).

The next several figures depict recombination traces and the corresponding MEM-derived kinetic populations associated with the bimolecular recombination under pseudo-first order conditions (high CO to heme ratio).

Figures 5a and 5b show the rebinding kinetics and corresponding MEM analysis respectively for solution phase COHb(N β 102A) as a function of added effectors. The MEM populations, displayed as the reciprocal of the rebinding rate, are divided into two major groupings. The faster groups which appears between 10^{-9} and 10^{-5} seconds, contains the kinetic phases associated with geminate recombination. The slower grouping which appears between 10^{-4} and 10^0 seconds contains those kinetic phases/populations associated with the so called solvent or bimolecular recombination phases. The distinct peaks shown in the MEM derived traces are referred to as kinetic populations to aid in distinguishing and classifying distinct groupings of kinetic constants. Several of the traces yield MEM derived populations that are between the geminate and bimolecular populations. These intermediate peaks are not as yet assigned but, based on similar studies on myoglobin mutants (86, 96), appear to be associated with rebinding from remote sites in the protein under conditions where the side chains of the distal hemepocket have not fully relaxed and water has not reoccupied the vacated distal hemepocket. These peaks and their assignment have no bearing on the assessment of T state populations which are based on the distribution of pseudo first order rate constants derived from the solvent phase bimolecular recombination processes. The multiple geminate phases as well as the intermediate phases are the focus of a future manuscript. T state bimolecular populations are further subdivided into high affinity T (HT) and three low affinity T (LT) populations. These groupings arise in part from the clustering of kinetic values obtained from the MEM analysis which are provided in Table 2.

Figures 5a and 5b show a clear pattern with respect to added effectors to the N β 102A mutant. With the addition of effectors, the geminate yield decreases and the bimolecular recombination gets slower. In each instance the kinetic trace is clearly not made up of a simple sum of T and R as seen for HbA in Trace c of Fig 4. The MEM-derived kinetic populations that correspond to each trace in Fig. 5a are shown in Fig. 5b. In Fig. 5b, Trace a shows that in the presence of both IHP and L35, the bimolecular rebinding contains contributions from all four T state kinetic populations (LT3 is seen as a shoulder (noted as (sh) in Table 2) on the “slow” side of the HT population. In the presence of only L35 (essentially the same trace with either IHP or L35), as seen in trace b, the population is now skewed further toward HT with a loss of LT1 and LT3. Trace c shows that in the absence of added effectors, the bimolecular phase is dominated by HT which can consistently (vide infra) be differentiated from the slightly faster R population observed for COHbA (trace d). The figure also shows that in going from a to d there is a progression of increasing relative amplitudes for the geminate phase with respect to the bimolecular phases.

Figures 6a and 6b display the kinetic traces and corresponding MEM analysis from sol-gel encapsulated samples of Hb(N β 102A) and HbA. Trace d, from HbA, is similar to the R state pattern seen from the solution phase but with an enhanced geminate yield due to the sol-gel(+25% glycerol)-induced increase in local viscosity (38, 97) and with a slow down in the

R state bimolecular phase. Also shown is the kinetic trace (trace c) for [COHbA+IHP] at pH 6.5. It can be seen that compared to [COHbA], there is a reduction in the geminate yield, as is also seen in solution (52), and a broadened R state bimolecular rate that is now skewed towards slower rates which the MEM shows to arise from a contribution from an HT population. At the extreme other end of the progression is trace a from [deoxy(N β 102A)+IHP]+CO which resembles previously reported T state traces from [deoxyHbA]+CO (32, 38, 97, 98) CO saturated Fe-Zn hybrids of HbA in either solution (plus effectors) or in sol-gel matrices (31). The trace from the corresponding encapsulated sample derived from the encapsulation of the CO derivative, i.e. [COHb(N β 102A)+IHP] is shown as trace b. It can be seen that relative to trace a from [deoxy(N β 102A)+IHP]+CO, the geminate yield is enhanced and the bimolecular phase is faster. Traces a and b in Fig. 6b show that the MEM populations display a loss of the HT peak and clear skewing of rates towards the LT populations for [deoxyHb(N β 102A)+IHP]+CO vis a vis [COHb(N β 102A)+IHP]. This result is consistent with encapsulation “locking-in” the initial conformational distribution and, as a consequence, the [COHb] and [deoxyHb]+CO samples will manifest the functional and spectroscopic properties of ligand-saturated and ligand-free distributions of conformations respectively. From Table 2 it can also be seen that when comparing the same COHb(N β 102A)+IHP sample in solution and in the sol-gel, the kinetic phases for the encapsulated sample are faster as is clearly seen for the HT population (420 versus 825 microseconds). These differences are explainable in terms of the sol-gel limiting the extent of tertiary relaxation subsequent to photo-dissociation and as a consequence the rebinding kinetics are reflective of the initial “more reactive” conformation for the CO derivative. In contrast, the solution phase sample will undergo tertiary relaxation that will slow the relaxation process (99).

Kinetic traces and corresponding MEM analysis for the CO recombination of a series of encapsulated samples of XLHb(W β 37E) are shown in Figures 7a and 7b respectively. It can be seen that for XLHb(W β 37E), the kinetic distribution for both encapsulated CO+IHP (trace c) and encapsulated deoxy + IHP turned CO (trace b) both manifest kinetic populations heavily skewed towards the HT regime with the latter showing evidence of a minor contribution from LT populations. It can be seen in Table 2 that the corresponding solution phase samples for the CO derivative with and without IHP, manifest slower kinetic traces with a single broad kinetic population in the LT3 regime. This difference between the solution and sol-gel samples is again explainable in terms of the rate of fast tertiary relaxation subsequent to photo-dissociation as discussed above for COHb(N β 102A)+IHP.

In contrast to fully liganded solution phase and encapsulated samples of XLHb(W β 37E) whose kinetic traces contain primarily the LT3 or HT populations respectively, the encapsulated half-liganded Fe-Zn hybrids of derivatives of XLHb(W β 37E) manifest kinetic traces that contain contribution not only from HT and LT3 but also from the slower LT2. This effect is seen in the representative trace a in Fig. 7b and in Table 2. It can be seen in Table 2, that the distribution of kinetic populations for the deoxy turned CO derivative for each of the Fe-Zn hybrids of this mutant has a higher fraction of LT population than the corresponding CO encapsulated derivative. Thus there is a clear progression in going from symmetrically half liganded to fully liganded derivatives of this mutant with respect to accessing the HT population. The results also indicate that for this hemoglobin, unlike Hb(N β 102A) and HbA (vide infra), sizable LT populations can not be generated for encapsulated samples of fully liganded derivatives even when starting with [deoxyXLHb(W β 37E)]. For this mutant, unlike HbA and Hb(N β 102A) sizable LT populations for sol-gel samples can only be achieved when encapsulating partially liganded derivatives of this mutant. The results are consistent with the barrier for the ligand binding induced accessing of the HT population being much lower than for HbA or Hb(N β 102A) as also implied from crystallographic studies (30).

The question arises as to whether HbA can access the same HT/LT kinetic populations observed for either Hb(N β 102A) or Hb(W β 37E). Figures 8a and 8b display several representative traces and MEM populations derived from [deoxyHbA]+CO. Trace a is derived from a [deoxyHbA]+CO sample that was allowed to age for an extended period prior to addition of CO (labeled [dxHb aged]+CO in Table II). The MEM analysis clearly reveals the three LT populations that are also evident in the corresponding Hb(N β 102A) traces. Trace b is derived from a sample prepared using a protocol (32) that allows for a slightly more rapid evolution of conformation upon addition of CO (labeled [dxHb, LT]+CO in Table 2). The corresponding MEM analysis reveals a substantial HT component. Trace c is derived from a sample prepared using a protocol (32) that allows for a much more rapid conformational evolution. This sample was allowed to extensively evolve over time (labeled [dxHb, HT]+CO in Table 2). It is a near endpoint trace where the sample has achieved the rebinding phase associated with the R state. Table 2 contains the MEM populations from these deoxy turned CO encapsulated HbA samples as well as those derived from a [deoxy HbA+IHP+L35]+ CO sample at one and eleven days after addition of CO. The pattern shows the evolution of the HT population towards faster rates and increasing amplitude at the expense of the LT populations. The range of rates for the HT population derived from [deoxyHbA]+CO samples covers the range seen for the various preparations of the two mutants discussed above.

Table 2 also contains MEM analysis of previously reported (31) kinetic traces from half liganded Fe-Zn hybrids in solution and in sol-gel. None of the traces manifest HT populations. For the α Zn/ β Fe hybrids, the addition of allosteric effectors shifts the distribution towards the slower LT populations; whereas for the α Fe/ β Zn hybrids, the addition of effector merely slows the single LT2 population. As with the mutants, encapsulation results in distributions that are consistent with a slowing of tertiary relaxation as reflected in the presence of faster populations when compared to the corresponding solution phase sample.

A comparison of the MEM derived populations shown in Table 2 shows that the MEM populations observed for CO rebinding kinetics from Hb(N β 102A) and Hb(W β 37E) are also observed for either HbA intermediates trapped in the sol-gel or partially liganded HbA derivatives. An essential difference is that the various combinations of HT and LT populations are readily stabilized for the tetra-liganded CO derivatives of the two mutants, whereas, for HbA, accessing these liganded T state populations requires either trapping of intermediates via the sol-gel or partial ligation via the symmetric Fe-Zn hybrids.

Discussion

Conformational variability within the deoxy T state of Hb

The present study as well as several earlier studies (31, 32, 52) show that the frequency of $\nu(\text{Fe-His})$ band for deoxyHbA is stable at $\sim 214\text{--}215\text{ cm}^{-1}$ regardless of whether effectors are present or absent. Thus it is reasonable to assume that this frequency, also seen for the deoxy derivative of Hb(N β 102A), is indicative of one endpoint for accessible T state conformations. This stable endpoint spectrum of the equilibrium population of deoxyHbA is associated with a UV resonance Raman spectrum that indicates that the T state constraints associated with the hinge and switch regions of the $\alpha_1\beta_2$ interface are fully intact.

Deviations from this endpoint deoxy T state spectrum are not commonly seen for deoxy derivatives of most cooperative hemoglobins but are invariably seen for those deoxy T state Hbs with chemical or mutagenic disruptions to the so-called Trp β 37 cluster of residues in hinge region of the $\alpha_1\beta_2$ interface (26, 35). The hydrogen bond network that couples these residues stabilize a conformational arrangement that keeps the indole of β_2 37W rigidly

locked in its standard deoxy T state position. Perturbations that loosen the packing of the $\beta 37$ side chain result in an increase in the frequency of $\nu(\text{Fe-His})$ as seen for HbMontefiore (D $\alpha 126$ Y) (100), desArg ($\alpha 141$)Hb (64, 101) and mutants of HbA in which Tyr $\alpha 140$ is replaced with Phe, Ala or Gly (93). This effect was most compellingly demonstrated for a series of deoxy $\beta 37$ Hb mutants in which the X-ray crystallographic data showing a progressive loosening of the hinge region within the T quaternary state correlated with the progressive increase in the frequency of $\nu(\text{Fe-His})$ (36) which likely correlates with changes in the influence of Tyr $\alpha 140$ on the proximal heme linkage (93). In the present study, the deoxy derivative of XLHb(W $\beta 37$ E) falls into that category, having a frequency of $\sim 218 \text{ cm}^{-1}$ for $\nu(\text{Fe-His})$ both in solution and in the sol-gel.

Conformational properties of liganded T state

A $\nu(\text{Fe-His})$ frequency of $\sim 222 \text{ cm}^{-1}$ is observed for the 8 ns photoproduct of COHb(N $\beta 102$ A) in the presence of effectors at low pH. A similar frequency is seen for the early time photoproduct of liganded hemoglobin derivatives that have been unambiguously assigned as T state species. These would include NOHbA+IHP at low pH (43, 46), COHbKansas+IHP at low pH/high concentration (102), and COFe-Zn hybrids of HbA in the presence of allosteric effectors at low pH (31). For the Hb(N $\beta 102$ A) mutant, as with the Fe-Zn hybrids and NOHbA, these conditions eliminate cooperativity and yield a very low affinity T state species. This frequency is again observed for the photoproduct of [deoxyHbA]+CO shortly after the addition of CO (32, 42) under conditions where the resulting sample has functional properties characteristic of what has been termed the low affinity T state (28, 32). In the present study, it is seen that under similar encapsulation conditions, the [deoxyHb(N $\beta 102$ A)]+CO samples yield frequencies that are initially intermediate between that of the deoxy derivative (214 cm^{-1}) and end point values that are close to those of the above mentioned liganded T state species. Similar intermediate values are seen (unpublished results) for [deoxyHbA]+CO under conditions where the bathing buffer for the gel is extremely viscous. Thus for hemoglobin populations that are designated as low affinity T state, there is a bounded range for the frequency of $\nu(\text{Fe-His})$ which starts at 214 cm^{-1} for the deoxy derivatives and ends at $\sim 222 \text{ cm}^{-1}$ for the photoproduct of the liganded derivatives.

The photoproduct of liganded hemoglobins that are assigned as high affinity T state species exhibit a frequency for $\nu(\text{Fe-His})$ that is higher than that seen for the low affinity T state species. Previously, this higher frequency was demonstrated for [deoxyHbA]+CO samples prepared using an encapsulation protocol that produces HT populations (32). In the present study, the high affinity T state populations derived from encapsulated Hb(W $\beta 37$ E) also yield the higher frequency at $\sim 225 \text{ cm}^{-1}$.

The UV resonance Raman results obtained in the present study as well as earlier studies on Fe-Zn hybrids (31), encapsulated HbA (32) and $\alpha 140$ Y mutants of HbA (93), also exhibit a progression that tracks with the decrease in proximal strain. With decreasing proximal strain (as reflected in the increasing frequency of $\nu(\text{Fe-His})$), the UVRR spectra show a weakening of T state markers associated with the $\alpha_1\beta_2$ interface with the most systematic behavior associated with the hinge region as reflected in the behavior of bands sensitive to Trp $\beta 37$. The emerging pattern is consistent with both a distribution of accessible T state species and a tight coupling of proximal strain with the status of the hinge region of T state $\alpha_1\beta_2$ interface. These results closely match the recent conclusions of the Arnone group (30) based on a systematic X ray crystallographic study of the progression of global and local changes seen within T state crystals as a function of ligation for LT and HT species.

Distinct functional T states exposed using MEM

The kinetically distinct populations derived from the MEM method of extracting kinetic lifetimes ($1/k$) for the bimolecular solvent phase under pseudo-first order conditions (large excess of CO relative to Hb), reveals multiple kinetic populations associated with liganded T state species. These kinetic populations can be roughly grouped on the basis of the distribution of kinetic lifetimes $1/k$ for the pseudo first order solvent phase CO recombination process. The same distinct populations are seen for different samples but with differing amplitudes. As shown in Table 2, these populations have been somewhat arbitrarily grouped into three low affinity T populations (LT), one high affinity T state population (HT) and the R state population. For each grouping there is a bounded range of observed lifetime values. The preliminary results from new studies of how the MEM T state populations evolve for [deoxyHbA]+CO indicate that with time subsequent to addition of CO, there is a progression within each T state grouping from slower to faster rebinding times.

The distribution of the kinetic populations tracks with the photoproduct frequencies of $\nu(\text{Fe-His})$. The samples with the lowest frequency for $\nu(\text{Fe-His})$ yield kinetic populations heavily skewed towards the LT's with the longest lifetimes (slowest rebinding rates). There appears to be a clear progression where the increase in frequency is associated with either the appearance of faster populations or increases in the relative amplitude of the faster populations. The results suggest several trends. These include: i.) for a given species, the addition of effectors usually shifts the distribution towards the slower LT population; ii.) for [deoxyHb]+CO samples, encapsulation protocol allows for manipulation of the distribution of LT and HT populations contributing to the kinetic traces at early times subsequent to the addition of CO; iii.) for a given T species, when comparing the populations of a T state [COHb] sample with that of the corresponding [deoxyHb]+CO sample at early times, the populations associated with the former are biased towards HT and the faster LT populations, iv.) for a given set of solution or encapsulation conditions, the T state sample with the lowest proximal strain and highest oxygen affinity, will contain the highest fraction of HT population and v.) the progression from deoxy LT to liganded LT to liganded HT is correlated with a loosening of constraints associated with Trp $\beta 37$ and Tyr $\alpha 140$.

Relating conformation and ligand reactivity within the T quaternary state

The present study shows there are several functionally distinct conformations that can be accessed within the T quaternary state. This observation is consistent with several recent assessments of the allosteric mechanism within hemoglobin that postulate multiple tertiary states within either T or R (12, 15, 98, 103, 104). A challenge in observing and characterizing these multiple T state conformations is that factors that favor the formation of the higher affinity T state conformations also favor the build up of R state population. In the present study sol-gel encapsulation and mutagenesis allow for the build up of higher affinity T state conformations under conditions where in solution with HbA, the population would be heavily biased towards the liganded R state conformations.

Fig. 9 depicts a hypothetical reaction coordinate diagram that provides a framework for understanding the ligand binding-induced changes within the T state. It is based on the present work which clearly shows that there are distinct populations within the T state family and that there is a clear progression of T state populations in response to ligand binding. Furthermore the slow diffusive nature of the evolution of structure within the sol-gel is suggestive of a "rough" energy surface as depicted in the figure which would be consistent with the proposed entropic search models (105, 106) that account for protein conformational changes.

The present study reveals two reaction coordinates for conformational change that need to be considered for HbA relaxation phenomena. One coordinate (the x axis) is for accessible conformations within the T state and thus describes the energy landscape that dictates the evolution of the T state tertiary structure upon ligand binding. In addition for each conformational substate or microstate within the T state there is a perpendicular coordinate shown as a red arrow that describes the transition from the T to R state. Upon ligand binding to the hemes, the T state population evolves from the now destabilized deoxy T state endpoint (extreme left of Fig. 9) towards the high affinity T state endpoint. The liganded T state intermediates represent a “loosened” structure suggestive of a transition state that provides a reduced barrier pathway for accessing the R state. It follows that as the population evolves towards the high affinity T state intermediates, the barrier for the T to R state gets lower (and the rate $k(T \rightarrow R)$ gets faster). In solution, the rate at which the R state is accessed is sufficiently fast to preclude the build up of measurable populations of the higher affinity T state species. Mutations such as N β 102A and W β 37E and sol-gel encapsulation can allow for the probing of the T state conformational coordinate by either destabilizing the R state sufficiently relative to the T state or by slowing the T to R transition. It has been proposed that the stabilization of hydration shell waters of Hb by the sol-gel plays a role in raising the barrier for the T to R transition (31, 38, 42) as this transition is associated with a large increase in the degree of hydration of the tetramer (107–112).

The distribution of T state conformations at any given time point subsequent to ligand binding appears to be the result of several competing forces acting on the tetramer. A framework for understanding these push-pull effects can be extracted from a model derived from several studies on the role of the segment of the hinge region of the $\alpha_1\beta_2$ interface that includes the Trp β 37 cluster. Recent research has demonstrated that the major region of quaternary-T constraint is derived from the coupling between the conformational status of the “Trp β 37 cluster” and the proximal strain at the α heme (26, 30, 35, 51, 93).

The present results imply that the stability of the Trp37 cluster also impacts the rate of conformational evolution. In the sol-gel, the Hb(W37E) mutant rapidly accesses HT upon ligand binding as do desArgHb and Hb(α 140Y) mutants (unpublished observations). In contrast, similarly encapsulated deoxy derivatives of HbA and Hb(N β 102A) in the presence of effectors especially L35, exhibit slow CO induced evolution away from the low affinity end point T state conformation consistent with a stabilization of the β 37 cluster.

A proposed stereochemical model: quaternary state modulation of tertiary conformations of the $\alpha\beta$ dimers

The present results are consistent with effector-induced modulation of tertiary structure impacting oxygen affinity (12, 104), fitting of T state kinetic data using a multi-tertiary structure model (98) and thermodynamic data indicating cooperativity within the T state (20, 21, 113). A promising new perspective that unifies these varied results and models is emerging from several lines of investigation. These include thermodynamic studies that indicate communication and a degree of cooperativity within $\alpha\beta$ dimers within the tetramer (20, 21, 114, 115) and functional/spectroscopic studies (116) on isolated dimers (semi Hbs) and most recently X-ray studies (30) that show that as the β 37 cluster is loosened there is a concomitant change in the bending of the individual $\alpha\beta$ dimers. The semi Hb study (116) shows that ligand binding favors tertiary structures for the $\alpha\beta$ dimer that are either R-like or HT-like, whereas the absence of ligand and/or the addition of effectors favors tertiary conformations that are more LT-like. However in both cases, the dimer does not access the extremes in either tertiary structure or functionality that are associated with the tetramer. Thus the conformational and functional parameters associated with both quaternary

constraint and quaternary enhancements are not achieved for the isolated dimers in spite of the large dynamic range of accessible conformations for the isolated dimers.

The present study is consistent with a picture in which the degree to which the dimer within the T state tetramer responds to ligation is governed by the stability of the Trp 37 β cluster. When the β 37 cluster/hinge is loosened, as occurs upon ligand binding within the T state, the dimer is no longer constrained and can now relax towards its preferred i.e. more stable conformation upon ligation even within the T quaternary state, as is also seen in the crystal study. The driving force for the switch to the liganded R structure now comes from the relative stabilities of the loosened T state structure and the liganded R structure under conditions where the $\alpha\beta$ dimer is liganded.

Conclusions

The use of mutant forms of HbA and/or sol-gel encapsulation to slow or eliminate the ligand binding-induced T $\rightarrow$ R transition has allowed for the spectroscopic and functional probing of different liganded T state conformations. The results of the present study indicate that there is a distribution of functionally distinct liganded T conformations and that the conformational composition of observed composite populations is modulated by a number of factors that include the stability of the hinge region of the $\alpha_1\beta_2$ interface and degree of ligation. The present results build on the earlier reports (28, 29, 32) on low and high affinity forms of the T state (LT and HT respectively). The present study shows that upon full and partial ligand binding, solvent conditions, encapsulation in sol-gel matrices and mutation can influence the distribution of LT and HT populations. The data are consistent with ligand binding to deoxy Hb initiating a progressive evolution both from LT to HT conformations and from slower to faster populations of LT and HT. There is associated with this progression, both a decrease in proximal strain/quaternary constraint and a weakening of the hinge interactions within the $\alpha_1\beta_2$ interface associated with the deoxy T state. The rate and extent of this relaxation appears to be a function of the stability of the so-called Trp β 37 cluster of residues within the hinge region of the $\alpha_1\beta_2$ interface as well as the presence or absence of allosteric effectors. The results are consistent with a picture in which the T quaternary state encompasses a range of accessible $\alpha\beta$ dimer conformations and that the distribution of dimer conformations observed for a given T state population is a function of the ligation and effector-dependent stability of the Trp β 37 cluster of residues. The results can also account for the large range of T state oxygen affinities associated with HbA in terms of the environment/effector-dependent extent to which the T state tertiary structure evolves subsequent to oxygen binding. The evolved liganded T state distribution of conformational states will determine the off rate of the bound oxygens which is the major determinant of oxygen binding affinity.

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Abbreviations

HbA	adult human hemoglobin
CO	carbon monoxide

deoxy	deoxygenated or ligand-free
LT	low affinity T
HT	high affinity T
VRR	visible resonance Raman
UVR	UV enhanced resonance Raman
MEM	maximum entropy method
GR	geminate recombination, GR
XL	cross-linked
IHP	inositol hexaphosphate

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Figure 1.

The low frequency portion of the Soret enhanced resonance Raman spectrum derived from: a. deoxy HbA, b. deoxy Hb(N β 102A) +IHP, c. 8 ns photoproduct of COHb(N β 102A)+IHP, d. the 8 ns photoproduct of COHbA+IHP and e. the 8 ns photoproduct of COHbA. All of the samples are in the solution phase at pH 6.5 maintained at ~ 4 C

Figure 2.

The low frequency portion of the Soret enhanced resonance Raman spectrum of sol-gel encapsulated hemoglobins as a function of ligation for the Hb(N β 102A) mutant, with spectra from encapsulated HbA presented for comparison. a. [deoxy HbA], b. [deoxy Hb(N β 102A)+IHP], c. [deoxy Hb(N β 102A)+IHP]+CO after 1 hour, d. [COHb(N β 102A)+IHP], e. [CO HbA].

Figure 3.

A comparison of the high frequency portion of the 229 nm excited UV resonance Raman spectrum of deoxy (solid line) and CO (dashed line) derivatives of the following samples: a. HbA in solution, b. Hb(N β 102A)+IHP in solution, c. encapsulated deoxy and CO derivatives of Hb(N β 102A)+IHP and d. encapsulated deoxy Hb(N β 102A)+IHP before and after addition of CO. In each instance, the deoxy-CO difference spectrum (solid –dashed lines) is shown below the two individual spectra. Both the W3 and Y8a bands have been independently normalized (hence the back slash dividing the spectra at approximately 1580 cm^{-1}) to better demonstrate the frequency differences for each of these two bands.

Fig. 4.

Traces depicting CO rebinding to photodissociated CO saturated hemoglobin derivatives at pH 6.5 at 3.5 C in solution displayed on a log-log plot of normalized absorbance versus time for the following samples: a. COHbA(α Zn/ β Fe) + IHP, b. COHb(N β 102A) + IHP and L35, c. COHbA under high photodissociation limit, and d. COHbA in the low photodissociation limit. Except for trace c all traces were generated under the low photodissociation conditions (no difference between the high and low limits for traces a and b).

5a

5b

Fig. 5.

a. CO recombination on a log-log plot for solution phase samples in the low photodissociation limit: a. COHb(N β 102A)+IHP + L35, b. COHb(N β 102A)+L35, c. COHb(N β 102A), d. COHbA). See text for experimental details.

b. The MEM (maximum entropy method) kinetic populations displayed as $1/k$ on a log time scale derived from CO rebinding traces from the following solution phase samples: a. COHb(N β 102A)+IHP+L35, b. COHb(N β 102A)+L35, c. COHb(N β 102A) and d. COHbA. The kinetic populations are grouped into two categories: geminate recombination (GR) and bimolecular recombination (BR). The focus of the present study is primarily on the BR (bimolecular recombination) populations which have been subdivided into an R state (R),

three LT (low affinity T state) and one HT (high affinity T state) population. As can be seen in Table 2, each of the LT and the HT categories covers a range of values. The labels in the figure provide a rough indication of where each of these variable populations appears. The origin and nature of the multiple geminate and intermediate phases (between ten and a hundred microseconds) seen in the figure are the focus of a future paper.

6a

6b

Fig. 6.

a. CO recombination on a log-log plot for encapsulated hemoglobin samples in the low photodissociation limit: a. [deoxyHb(N β 102A)+IHP]+CO, b. [COHb(N β 102A)+IHP], c. [COHbA+IHP], and d. [COHbA]. See text for nomenclature and experimental conditions.

b. The MEM (maximum entropy method) kinetic analysis displayed as $1/k$ on a log time scale derived from CO rebinding traces from the following samples: a. [deoxyHb(N β 102A)+IHP]+CO, b. [COHb(N β 102A)+IHP], c. [COHbA+IHP], and d. [COHbA].

7a

7b

Fig. 7.

a. CO recombination on a log-log plot for encapsulated hemoglobin samples in the low photodissociation limit: a [COHb(W β 37E: α Zn/ β Fe)+IHP], b. [deoxyXLHb(W(β 37E)+IHP)+CO], c. [COXLHb(W β 37E)+IHP], d. [COHbA]. See text for nomenclature and experimental conditions.

b. The MEM (maximum entropy method) kinetic analysis displayed as $1/k$ on a log time scale derived from CO rebinding traces from the following sol-gel encapsulated samples: a [COHb(W β 37E: α Zn/ β Fe)+IHP], b. [deoxyXLHb(W(β 37E)+IHP)+CO], c. [COXLHb(W β 37E)+IHP], d. [COHbA].

8a

8b

Fig. 8.

a. CO recombination on a log-log plot for [deoxy HbA]+CO as a function of preparative protocol: a. [deoxyHbA]+CO, the sample was extensively aged prior to addition of CO; b. [deoxyHbA]+CO, prepared using the LT protocol (32), c. [deoxyHbA]+CO prepared using the HT protocol (32), the trace was generated one month after addition of CO, d. [COHbA]. See text for nomenclature and experimental conditions.

b. The MEM (maximum entropy method) kinetic analysis displayed as $1/k$ on a log time scale derived from CO rebinding traces from [deoxyHbA]+CO samples as a function of time subsequent to the addition of the CO. a. [deoxyHbA]+CO, the sample was extensively aged prior to addition of CO; b. [deoxyHbA]+CO, prepared using the LT protocol (32), c.

[deoxyHbA]+CO prepared using the HT protocol (32), the trace was generated one month after addition of CO, d. [COHbA]. See text for nomenclature and experimental conditions. The asterisks designate a region where a peak was cosmetically removed (see text).

Fig. 9.

A hypothetical reaction coordinate diagram that depicts the proposed evolution of T state tertiary structure upon ligand binding. Perpendicular to this reaction coordinate is the reaction coordinate for the T $\rightarrow$ R transition. The numbered potential minima represent different T state species that are progressively accessed subsequent to ligand binding. For HbA, the initial deoxy population is dominated by the potential well labeled 1 which prior to ligand binding would be much deeper than shown. The progression from 1 to 5 represents the T state tetramer relaxation from the low affinity T (LT) to high affinity T (HT). In going from LT to HT (i.e. 1 $\rightarrow$ 5), the $\alpha_1\beta_2$ interface loosens, proximal strain decreases and the barrier for the T $\rightarrow$ R switch is reduced resulting in a progressive increase in the T to R rate constant. Thus for each T state conformation (designated 1–5) there is a corresponding T to R rate constant, $k_n(T_n \rightarrow R)$, with n being the designation for the specific T state. These rate constants increase as n goes from 1 to 5. Sol-gel encapsulation greatly slows $k(T \rightarrow R)$ relative to the LT $\rightarrow$ HT transition. The overall energy surface is portrayed as a rough energy landscape based on the diffusive-like evolution of conformation observed for the sol-gel samples.

Table 1Visible Resonance Raman data – Fe-His band peak position (cm^{-1})

Protein	deoxy			CO		
	+IHP	+IHP+L35	+IHP	+IHP	+IHP+L35	
HbA	214		230	228	224	
α Zn/ β Fe	220	220.5			222.5	
β Zn/ α Fe	205				221	
XL β 37E	217.5	217.5	224.5	224	224	
XL α Zn β W37E	222.5		224.5	224.5		
XL β Zn β W37E	216.6		225	224.5		
β N102A		214	226.5	223	221.5	
[dxXL β W37E]	218.5					
[COXL β W37E]			224.5	224.5		
[dxXL β W37E]+CO			224.5	224.5		
[CO β N102A]			223			
CO addition timeline	No CO	1 hour	2 day	9 day	14 day	36 day 5 month
[dx β N102A+IHP]	214	217	217.5	218	219	219.5 223.5

Table 2

MEM generated bimolecular CO recombination lifetimes for hemoglobins in solution and sol-gel under pseudo-first order conditions.

Sample	R (μs)	HT (μs)	LT3 (ms)	LT2 (ms)	LT3 (ms)
COHb	140 (1.0)				
[COHb]	280 (1.0)				
[COHb+IHP]	235 (0.71)	630 (0.29)			
COHb(Nβ 102A)	356 (1.00)				
COHb(Nβ 102A) +L35	414 (0.53)		7 (0.11)	37 (0.35)	
COHb (Nβ 102A)+IHP	825 (0.49)		13 (0.18)	43 (0.33)	
COHb (Nβ 102A) +IHP+L35	708 (0.29)		~2 (sb)	27 (0.33)	89 (0.38)
[COHb(Nβ 102A)+IHP]	420 (0.28)		2.5 (0.38)	33 (0.33)	
[dxHb (Nβ 102A)+IHP]			2.0 (0.39)	14 (0.32)	55 (0.28)
COXLHb(Wβ 37E)			1.0 (1.0)		
COXLHb(Wβ 37E)+IHP			2.5 (1.0)		
[COXLHb(Wβ 37E)+IHP]	455 (1.0)				
[dxXLHb(Wβ 37E)+IHP]	695 (0.64)		1.3 (0.35)		
[COHb(Wβ 37E:α Zn/β Fe)+IHP]	750 (0.52)			17 (0.48)	
[dxHb(Wβ 37E:α Zn/β Fe)+IHP]	675 (0.12)		2.4 (0.17)	17 (0.71)	
[COHb (Wβ 37E:α Fe/β Zn)+IHP]	630 (0.29)			16 (0.71)	
[dxHb(Wβ 37E:α Fe/β Zn)+IHP]			2.5 (0.34)	25 (0.66)	
[dxHb, aged]+CO			1.2 (0.25)	11 (0.43)	56 (0.31)
[dxHb, LT] +CO		564 (0.39)		22 (0.17)	
[dxHb, HT] +CO	202 (1.0)				
[dxHb+IHP+L35]+CO					
day1 *		768 (0.12)		11 (0.20)	44 (0.65)
day11 *		400 (0.55)		11 (0.24)	25 (0.22)
CO Hb (α Zn/β Fe)			1.4 (0.20)	17 (0.42)	139 (0.52)
CO Hb (α Zn/β Fe)+IHP				24 (0.28)	71 (0.59)
[CO Hb(α Zn/β Fe)+IHP]			1.0 (0.29)	10 (0.19)	33 (0.32)
COHb (α Fe/β Zn)				18 (1.00)	
CO Hb (α Fe/β Zn)+IHP				24 (1.00)	

Sample	R (μs)	HT (μs)	LT3 (ms)	LT2 (ms)	LT3 (ms)
[COHb (α ₁ Fe ²⁺ β ₂ Zn)]				11	(0.90)

Notes: Brackets [] indicate that the protein is encapsulated in a sol-gel. Absence of brackets indicates a solution phase sample. (See text for detailed nomenclature; dx is used to denote deoxy).

* denotes day/s post CO addition.

The data in the table are the centerpoints of the MEM distributions; values in red represent the fraction of normalized population for the kinetic bimolecular phase. Normalization was done for each sample, based on the relative amplitude of kinetic populations with bimolecular CO lifetimes greater than 100 μs.