more frequent for the vowel group (13 intrusions equaling .41 of all words recalled) than for the liking group (5 intrusions equaling .06 of all words recalled).

Our findings show a pattern of results with normal subjects that mirrors the performance of anterograde amnesic patients. Like amnesics, our vowel subjects gave little evidence of having seen the words that they successfully produced on the completion test. The vowel subjects explained, for example, that they "did not look at the words" or that they were "not instructed to look at the words." This dissociation of recall and completion performance is predicted by the dual process model of recognition for any group of subjects that is prevented from elaborative processing. Different and separate underlying processes are responsible for recall and for completion. The advantages of elaborative processing are not available to amnesic patients—they are unable either to construct and store or to retrieve the elaborative network necessary for recall. In the case of our vowel group, we have prevented elaborative encoding and produced similar results.

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1. For example, alcoholic patients with Korsakoff syndrome and patients who have received bilateral electroconvulsive therapy for the relief of depressive illness. For recent reviews of amnesics' memory deficits see L. R. Squire, N. J. Cohen, and L. Nadel [in *Memory Consolidation*, H. Weingartner and E. Parker, Eds. (Erlbaum, Hillsdale, N.J., in press)] and E. K. Warrington and L. Weiskrantz (*2*).
2. E. K. Warrington and L. Weiskrantz, *Neuropsychologia* **20**, 233 (1982).
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5. Warrington and Weiskrantz have recently revised their retrieval interpretation of amnesics' memory deficit (*2*). They distinguish between a semantic and a mediational (retrieval) memory system. Completion test performance depends on the semantic system, which is spared in amnesia.
6. See E. K. Warrington and L. Weiskrantz [*Nature (London)* **228**, 628 (1970)] and L. R. Squire [*Neuropsychologia* **18**, 369 (1980)] for critical discussions.
7. G. Mandler, *Psychol. Rev.* **3**, 252 (1980); *Am. Sci.* **69**, 211 (1981). For general evidence for the dual process model see also R. C. Atkinson and J. Juola [in *Contemporary Developments in Mathematical Psychology*, vol. 1, *Learning, Memory, and Thinking*, D. H. Krantz *et al.*, Eds. (Freeman, San Francisco, 1974), p. 243] and G. Mandler, G. O. Goodman, and D. L. Wilkes-Gibbs [*Mem. Cognit.* **10**, 33 (1982)].
8. In several pilot studies, we had used limited exposure (100 to 200 msec) compared with 1- to 2-second exposures. However, this manipulation did not produce the dissociation between recall and completion test performance that we found with the liking rating and the vowel-search task.
9. These instructions were designed to encourage subjects to produce words that were immediately accessible; the words whose activation level had been increased during the original presentation and that also satisfied the appropriate three-letter stems should be the most likely responses in completing the stems.
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Transient Raman Study of Hemoglobin: Structural Dependence of the Iron-Histidine Linkage

Abstract. *Low-frequency resonance Raman spectra of transient hemoglobin species were observed within 10 nanoseconds of photolysis. The Raman frequencies of the iron–proximal histidine stretching mode for transient species having either the R or the T quaternary structure are higher than in the corresponding deoxy species. The observed frequency difference in the iron-histidine mode between the R- and T-state transients indicates that there are quaternary structure–dependent protein forces on the iron-histidine bond in the liganded hemoglobins. These differences are interpreted in terms of changes in the tilt of the histidine with respect to the heme plane.*

Transient forms of hemoglobin (Hb) resulting from photodissociation of bound ligands provide a means of studying the conformational changes that initiate the events leading to the quaternary structure transition. After the photolysis of COHb, the porphyrin relaxes within picoseconds to a configuration nearly identical to that of an electronically relaxed deoxy (five-coordinate) heme (*1–6*). An important consideration in determining the mechanism for subsequent protein dynamics (such as the quaternary structure change) is the coupling mechanism between the nonequilibrium protein and the electronically relaxed deoxy heme. By comparing the resonance Raman spectrum of the deoxy heme in the transient species to that of the corresponding relaxed species, one can examine how specific degrees of freedom of the deoxy porphyrin are modified by the protein structure. The iron–proximal histidine (Fe–His) linkage is a potentially important element in models describing cooperativity in Hb. Using time-resolved resonance Raman scattering, we have determined the frequency of the Fe–His stretching mode from both high-affinity (R-state quaternary structure) and low-affinity (T-state quaternary structure) photodissociated ligand-bound hemoglobins. Compared to the relaxed deoxy species, the Raman bands associated with the transient species at 10 nsec are shifted to higher frequency by 6 to 10 cm^{-1}. In both the deoxy and the transient species, the Fe–His stretching frequency is higher in the R state than in the T state.

Transient resonance Raman spectra were generated with the output of a dye laser pumped by a nitrogen or an excimer laser operating at 10 Hz. The tunable output ($\leq$ 0.6 mJ) of the dye laser consisted of 10-nsec pulses. An excitation frequency near 4200 Å (Bis MSB) or 4350 Å (stilbene 3) was used to achieve resonant enhancement of the relevant Raman bands. The excitation light was focused (with a lens of focal length 250 mm) into a temperature-controlled cuvette at 2°C. No differences in spectra were observed between static and recirculated samples. The 90° scattered light was collected by an off-axis elliptical mirror and dispersed by a 1-m *f*/8 J. Y. Ramanor HG-23 spectrometer fitted with an RCA C313034-02HQ photomultiplier tube. The output of the photomultiplier tube was gated (1 nsec) and averaged with a PAR model 163 boxcar integrator. The resulting signal was stored and processed with a Nicolet 1174 signal averager, which allowed for repetitive scans of a spectrum (reset error < 0.2 cm^{-1}). A custom-designed interference filter for the excitation pulses was used to reduce the fluorescence background associated with the output of the dye laser.

The R-state transient was derived from photolyzed human adult COHb and NOHb and the corresponding T-state species were derived from photolyzed NOHb and from COHbK (Kansas) (700 μM, *p*H 6.5, 100 m*M* bis-tris), both in the presence of inositol hexaphosphate (IHP). The high heme concentration was necessary to ensure a sizable population of tetramers in the HbK (Kansas) sample. No spectral dependence on heme concentration (100 to 500 μM) was observed for the R-state transients.

Figure 1 shows low-frequency resonance Raman spectra of transient Hb species occurring within 10 nsec of photolysis of COHb. The upper and lower spectra are from the photolyzed carboxy derivatives of adult hemoglobin (HbA) and HbK (plus IHP, *p*H 6.5), respectively. Under the conditions used, the transients associated with HbA and HbK have the quaternary structure of the R state and the T state, respectively. From

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these spectra the frequency associated with the Fe–His stretching mode is ~ 230 cm^{-1} for the R-state transient species and ~ 222 cm^{-1} for the T-state transient species. Approximately the same frequencies for T and R states, respectively, were found for NOHb with and without IHP (Table 1). The corresponding frequencies (*7, 8*) in equilibrium deoxy HbA are 216 cm^{-1} (T state) and 222 cm^{-1} (R state derived from genetically or chemically modified Hb).

From the behavior of the Fe–His stretching modes, we conclude that the transient species has a stronger Fe–His bond than the deoxy counterparts; specifically, within each quaternary structure the Fe–His stretching mode is at a higher frequency for the transient. Therefore for both quaternary structures, ligation induces substantial structural changes about the heme. Baldwin and Chothia (*9*) concluded that the positioning of the histidine is primarily a consequence of the quaternary structure irrespective of the presence of bound ligand. However, the results reported here indicate that the positioning of the histidine is controlled by both quaternary structure and ligation.

Although different ligands are known to generate slightly different structures (*10*), for a given quaternary structure in the transient species at 10 nsec the Fe–His frequencies are indistinguishable. For NOHb plus IHP there is strong evidence that the Fe–His bond is either severed or severely weakened (*11, 12*). However, at 10 nsec the Fe–His frequency is the same as that of photolyzed COHbK, in which there is no evidence for an anomalous Fe–His bond. Furthermore, despite substantial differences in the iron-to-center distance in COHb between the α and β subunits (Table 1), we detect no chain-specific differences in the transients. These results indicate that, although the protein retains memory of the liganded state, the ligand-specific features have relaxed within 10 nsec of photolysis.

The question remains whether, within 10 nsec, the Fe–His bond length has achieved its equilibrium "deoxy" value, which would allow a direct structural comparison of the Fe–His linkage between the equilibrium and transient species. Central to this question is when the in-plane iron of the liganded heme reassumes an out-of-plane position characteristic of the deoxy heme. There is theoretical (*13–16*) and experimental (*17*) evidence that in a five-coordinate heme, nonbonded interactions between the proximal histidine and the pyrrole nitrogens of the porphyrin macrocycle strongly favor an out-of-plane iron. Calculations (*16*) show that the nonbonded repulsive energy drops from 15 to 6 kcal when the iron atom moves to 0.3 Å out of plane. If the iron in the transient remained in plane, as suggested (*5, 6*) on the basis of other transient Raman studies (*5, 6, 18*), then to account for the increase in frequency the protein would have to generate a force counter to the sizable repulsive force discussed above; otherwise the iron would be expected to assume an out-of-plane configuration within a few vibrational periods (less than picoseconds). The observed increase in frequency would have to be explained in terms of this postulated restraining force inducing a shortening of the Fe–His bond. The rigidity that would be necessary for these forces is not evidenced in crystallographic (*9*) or theoretical studies (*13, 14, 19*). Furthermore, if the iron maintained the same position in this transient species as in the six-coordinate hemoglobin, the Raman frequency should be correlated with the known displacements in the liganded species. As seen in Table 1, there is not such a correlation. This is especially notable in the comparison of COMb (carboxymyoglobin) and the β subunits of COHb, which have identical iron displacements but very different transient Raman frequencies.

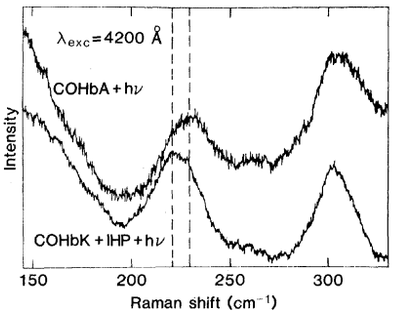

Fig. 1. The low-frequency resonantly enhanced Raman spectrum of the transient deoxy species occurring within 10 nsec of photodissociating carboxyhemoglobin. The unliganded species giving rise to the upper spectrum has the R quaternary structure whereas that giving rise to the lower spectrum has the T structure. The spectrum associated with HbK has a shoulder on the high frequency side of the 222 cm^{-1} band which is attributed to ≈ 15 percent of the heme population which exists as dimers under these experimental conditions.

Movement of the iron out of the heme plane should be accompanied by substantial changes in both Raman and absorption spectra. Transient absorption (*4*) and Raman studies (*5, 6*) show a major change in the electronic and nuclear structure of the heme within a few picoseconds of photolysis, followed by an unchanging spectrum from 1 to 20 nsec. Recent pulse-probe transient Raman studies (*20*) indicate that the Fe–His stretching mode in various photolyzed Hb's relaxes on the time scale of hundreds of nanoseconds to hundreds of microseconds. Thus either the iron relaxes out of plane in the predicted picoseconds or less or remains in plane for at least a factor of 10^5 to 10^7 longer than the predicted subpicosecond time scale for model heme systems. On the basis of the above considerations, we assume that the transient at 10 nsec does have an out-of-plane iron.

Even though we infer that in 10 nsec

Table 1. Frequencies of the Fe–His stretching mode from a variety of high-affinity and low-affinity forms of deoxyhemoglobins and photodissociated (10 nsec) hemoglobins. Also shown are the known values for several structural parameters associated with both the displacement of the iron and the distances between the carbons of the proximal histidine and the nitrogens of the heme. The Fe-center distance is the distance between the iron atoms and the center of the pyrrole nitrogen core. The structural parameters refer to the unphotolyzed species.

Sample	State	Fe–His frequency (cm^{-1})	Fe-center distance (Å)	His-heme distance (Å)	
				C^{ε}-N^{1}	C^{δ}-N^{3}
Deoxy HbA					
α subunit	T	201/212*	0.60 (*27*)	3.2 (*9*)	3.8 (*9*)
β subunit	T	218*	0.63 (*27*)	3.5 (*9*)	4.2 (*9*)
Deoxy NES des-$Arg^{141\alpha}$-HbA†	R	222 ± 2			
Deoxy Mb		222 ± 2	0.42 (*27, 28*)	3.6 (*28*)	3.5 (*28*)
COMb		222 ± 2	0.18‡	3.3‡	3.2‡
COHb (Kansas) + IHP + hν	T	222 ± 2			
NOHb + IHP + hν	T	222 ± 2			
COHbA (*p*H 9.0) + hν	R	231 ± 1			
α subunit	R	231 ± 1§	0.04 (*29*)	3.1 (*9*)	3.4 (*9*)
β subunit	R	231 ± 1§	0.22 (*29*)	3.2 (*9*)	3.2 (*9*)
COHb (Kempsey) (*p*H 9.0) + hν	R	231 ± 1			
NOHb (*p*H 9.0) + hν	R	231 ± 1			

*Values for valence hybrids (*7*) and for Fe-Co hybrids (*30*). †Value from (*7*); NES, (*N*-ethylsuccinimido)-cysteinyl; Arg, arginine. ‡Distance for O_2Mb from Phillips (*31*). §Values for Fe-Co hybrids from Friedman *et al.* (*32*).

the iron has relaxed to an out-of-plane position, our data still have implications regarding the forces in the liganded protein. The R-T differences in the transient spectra indicate that there are quaternary structure–dependent protein forces on the Fe–His bond in the liganded hemoglobins. This follows because the protein quaternary structure relaxes on a much longer time scale (*21–23*). To translate these differences into energies in the six-coordinate case, it is necessary to know the R-T–dependent forces responsible for the variation in Raman frequency. As a first step we have attempted to correlate known structural features with the observed frequencies.

The differences in frequency between the deoxy and transient species could originate from a variety of sources. One possibility is that 10 nsec after photolysis the heme is in a long-lived excited electronic configuration which affects the force constant of the Fe–His stretching mode. This is unlikely because (i) there is no spectral evidence for such an excited electronic state and (ii) the time evolution of this mode after photolysis is characteristic of a protein nuclear coordinate relaxation rather than an electronic relaxation at the heme. For example, at room temperature the frequency relaxes in microseconds (*20*), whereas at cryogenic temperatures the transient can be stabilized for hours (*24*). We therefore assume that the frequency differences in the steady-state species and the transients originate from the influence of the protein on the Fe–His linkage. We examined crystallographic data in search of a structural parameter that can, within the uncertainties of the x-ray data, account for the observed frequency behavior of the Fe–His mode in a consistent and straightforward fashion. The protein degree of freedom that satisfied these criteria is the tilt of the proximal histidine with respect to the heme plane. Theoretical considerations (*14–16*) also indicate that this tilt is important in regulating ligand binding. In the following we examine the relation between this tilt and the frequency of the Fe–His stretching mode.

The tilt of the histidine (in its own plane) with respect to the porphyrin plane results in a difference in the distances (see Table 1) between the two pairs of imidazole carbons and the pyrrole nitrogens. In going from the liganded to the deoxy case, the histidine in myoglobin retains its symmetric orientation, whereas in hemoglobin it goes from a symmetric to a tilted orientation. This small tilting has minimal kinematic effects (*25*), but it should weaken the Fe–

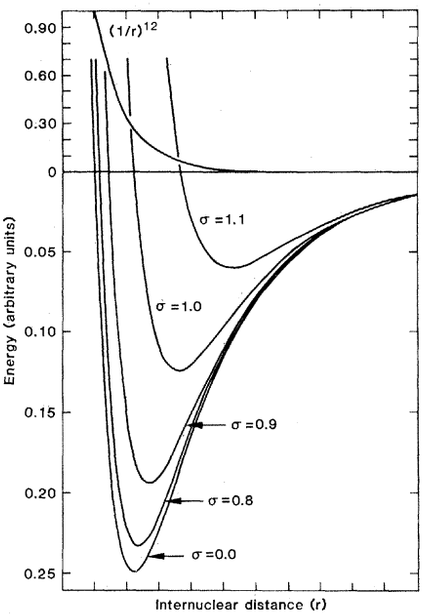

Fig. 2. Schematic two center potential energy surfaces generated by the addition of a repulsive term $(\sigma/r)^{12}$ to a Lennard-Jones potential $[(l/r)^{12} - (l/r)^6]$. The scale on the top for the repulsive potential has one tenth the sensitivity of the scale used for the Lennard-Jones potentials.

His bond and thereby lower the Raman frequencies because of the increase in nonbonded interactions (*14*). A hypothetical diatomic Lennard-Jones potential for this bond is shown in Fig. 2. An increased nonbonded interaction for the slightly tilted conformation may be described by adding a $(1/r)^{12}$ repulsive term to the Lennard-Jones potential. Figure 2 shows a range of such repulsive terms, which cause the original potential function to become shallower and less harmonic (*26*). Thus, in the absence of kinematic effects, stronger repulsive terms result in lower vibrational frequencies. If the change in Raman frequency is due to a change in nonbonded interactions, there should be no difference between deoxy and photolyzed myoglobin, whereas in hemoglobin the frequency should decrease in going from the photolyzed R-state conformation with an untilted histidine to the deoxy T-state conformation with the most tilted histidine. In addition to the histidine tilt, other protein degrees of freedom within the heme-histidine unit can contribute to the baseline Fe–His bond strength, which makes interprotein comparisons difficult to analyze in terms of a single parameter. For example, differential displacement of the iron from the heme plane in hemoglobin and myoglobin could account for the lower Raman frequency of myoglobin relative to photolyzed R-state hemoglobin even though both have untilted histidines. Nonetheless, within the hemoglobin system, it seems highly plausible that protein– and ligand-binding–induced modulation of the tilt angle accounts for most of the change in the Raman frequency between deoxy and photolyzed species. We conclude from this first-order analysis that the ordering of hemoglobin species with respect to the tilt of the histidine is: deoxy T > photolyzed T ≈ deoxy R > photolyzed R.

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