The Absence of "Heme-Heme" Interactions in Hemoglobin

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Hemoglobin, the oxygen-transporting protein of blood, is composed of two pairs of subunits, the α- and β-chains. Each subunit contains a single heme, which can combine reversibly with oxygen so that each hemoglobin molecule can bind as many as four oxygen molecules. The affinity of hemoglobin for oxygen increases as more oxygen is bound, and the explanation of this "cooperativity" has been sought for many years. In this article we present results of nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR) experiments which show that no changes are detected in the heme groups themselves when neighboring heme groups are ligated. We also show that ligand-induced conformational changes do occur in myoglobin, which resembles a hemoglobin subunit, and we have indications of similar tertiary structural changes within the subunits of hemoglobin. These, it is suggested, lead to the cooperativity. The free-energy changes responsible for the cooperative oxygen binding come from the dependence of the interaction energy in the protein moiety of the subunits upon the degree of oxygenation. Hence *subunit interaction* is a more suitable term than *heme-heme interaction* to describe the cooperativity.

Excellent reviews of the research on ligand binding to hemoglobin are available. In particular, the investigations made before 1964 are reviewed by Rossi-Fanelli, Antonini, and Caputo (*1*); in the same volume appears Wyman's (*2*) definitive analysis of the properties of linked functions in proteins, particularly in hemoglobin. To emphasize the cooperative nature of oxygen binding to hemoglobin, a comparison should be made with myoglobin (Mb), which contains one heme and which binds one mole of oxygen per mole of protein, according to the reaction

$$\mathrm{Mb} + \mathrm{O_2} \rightleftarrows \mathrm{MbO_2} \qquad (1)$$

Hence, in the case of Mb, oxygen binding follows the hyperbolic curve predicted for bimolecular reactions of noninteracting binding sites. On the other hand, hemoglobin, which contains two α- and two β-chains, cooperatively binds four oxygen molecules, so the oxygen-binding curve has the familiar sigmoidal shape. From measurements of the binding of oxygen to sheep hemoglobin, Wyman has calculated that, to saturate hemoglobin with oxygen, the total free energy of interaction liberated, ΔF_I, is 3.0 kilocalories per mole. This interaction energy is about 10 percent of the value of the total free energy change during oxygenation of hemoglobin in solution. In order to differentiate between various possible contributions to ΔF_I, it is conceptually helpful to write

$$\Delta F_I = \Delta F_{IH} + \Delta F_{IP}$$

where ΔF_{IH} and ΔF_{IP} refer to the free energy contributions of the heme groups and the protein parts, respectively. From the NMR and EPR measurements on the heme groups of hemoglobin presented here, we have tried to evaluate the relative contribution of ΔF_{IH} to ΔF_I.

When hemoglobin is oxygenated, structural changes occur. In 1938, Haurowitz (*3*) observed that crystals of deoxygenated hemoglobin shatter when the hemoglobin is oxygenated, and he suggested that the shattering indicated structural changes. Considerable biochemical evidence for this structural change was accumulated before the structural change was clearly demonstrated by Perutz's (*4*) beautiful x-ray crystal-structure determination. These studies at a resolution of 5.5 angstroms, for both oxygenated and deoxygenated hemoglobin, showed that, upon oxygenation, the two β subunits roll upon the α subunits to form different $\alpha\beta$ contacts, thereby changing the quaternary structure of the molecule.

Two kinds of questions can be asked about this change in molecular structure upon oxygenation. First, what contribution does this structural change make to ΔF_I? Is it a negligible contribution, in which case ΔF_{IH} dominates ΔF_I and one would expect large changes at one heme group as its neighbors are oxygenated? Or is ΔF_{IP} large, so that the main contributions to ΔF_I are from the protein? Note that ΔF_{IP} can include contributions from the quaternary structural changes at the subunit interfaces as well as from tertiary structural changes within the protein moiety of the subunits.

The second kind of question concerns the origin of this structural change. Is it triggered by ligand-induced conformational changes? Or does it arise because oxygenation shifts the equilibrium between two forms (*5*) of hemoglobin which exist in the absence of oxygen? Answers to these questions are provided by the NMR and EPR experiments discussed below.

High-resolution proton NMR is particularly useful in studying heme proteins (*6*) because of two mechanisms which operate to increase the resolution of the spectra. The first depends upon the magnetic moment of the unpaired electrons delocalized around the porphyrin ring, which shift the NMR lines of the porphyrin protons by hyperfine interactions (*6, 7*). In this way, the proton resonances of the heme group are well separated from those of the polypeptide chains. Hyperfine shifts of proton resonances are inversely proportional to the absolute temperature, and the temperature-dependence of the shifts makes it possible to identify hyperfine shifted lines. The magnetic field at which an NMR line appears may shift as much as 100 parts per million as a result of a hyperfine interaction.

Ring-current shifts, the second useful interaction, arise from the local magnetic fields of aromatic residues near the observed protons, the largest effects coming from the porphyrin ring (*8*). These shifts are temperature-

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independent and thereby are experimentally distinguishable from hyperfine shifts. Ring-current shifts are very sensitive to the relative positions of the hydrogen nuclei whose resonances are observed and the aromatic ring responsible for the shift. Hence they are sensitive indicators of the conformation and conformational changes of a protein.

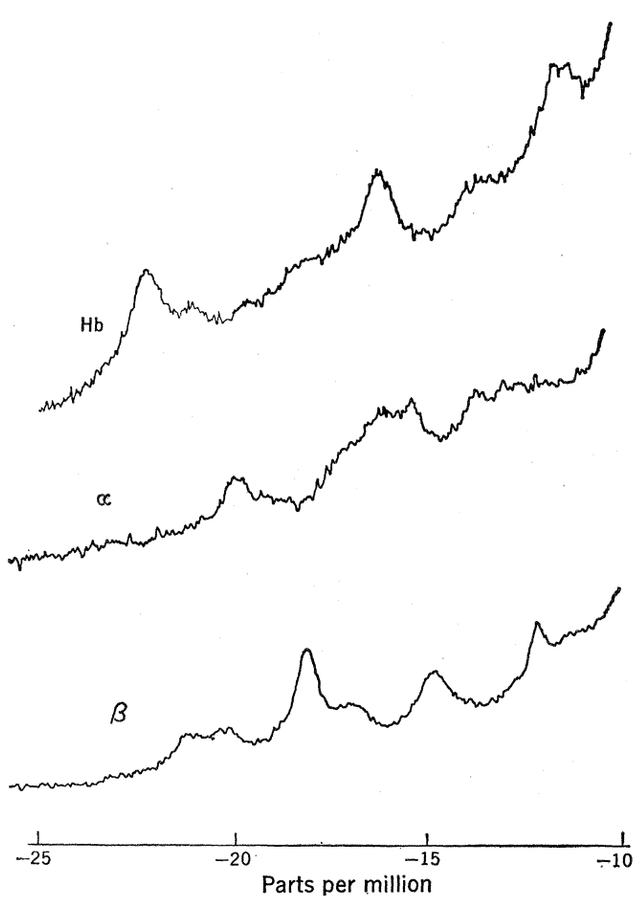
The EPR signal of high-spin ferric hemoglobin can be observed (*9*) in frozen aqueous solutions at low temperatures. If the iron is in a site of tetragonal symmetry, where the x and y axes in the porphyrin plane are indistinguishable, the spectrum consists of a weak line at an external magnetic field corresponding to a g-value of 2.0 and a strong line at $g \approx 6$. In sites of lower symmetry, where the x and y directions differ, the $g \approx 6$ resonance broadens, or it may even split into two lines in more extreme cases.

In the experiments discussed here, we use these NMR and EPR properties as indicators of changes in the vicinity of the heme group. Both types of magnetic resonance experiments, insofar as their results are sensitive to changes of the electronic properties of the heme, complement optical absorption measurements of the heme groups.

Experimental Methods and Materials

The NMR spectra were measured at 220 megahertz with a Varian HR-220 spectrometer. The signal-to-noise ratios were improved by averaging with a Fabritek 1062 computer. The volumes of the samples studied by NMR were 0.3 to 0.5 milliliter, and the heme concentrations were approximately $6 \times 10^{-3} M$. The resonance positions are relative to the internal standard DSS (3,-3-dimethyl-3-silapentane-5-sulfonate). The EPR spectra were obtained at 1.4°K in an x-band superheterodyne spectrometer operating at a frequency near 9500 megahertz. Samples (volume, 0.6 milliliter) were pipetted into the bottom of a rectangular silvered-glass EPR cavity and were frozen quickly by immersion in liquid nitrogen.

Human oxyhemoglobin A was prepared from freshly drawn blood by the method described by Bunn and Jandl (*10*). Beta chains were prepared as oxyhemoglobin H (β_4) (*11*). Oxyhemoglobin was separated into individual chains with parachloromercuribenzoate (PMB) by the method of Bucci and Fronticelli (*12*). The PMB was removed from the oxy-α-chains by absorption on a column of sodium carboxymethylcellulose (CMC) and washing with mercaptoethanol (*13*). The oxy-α-chains were then eluted from the column, and for samples of ferric α-chains they were quickly oxidized in the cold with excess ferricyanide and separated from excess oxidant through absorption on a Sephadex G-10 gel filtration column. More than 85 percent of the sulfhydryl groups of the isolated ferric α-chains were found to be intact after the oxidation, as measured by the method of Boyer (*14*).



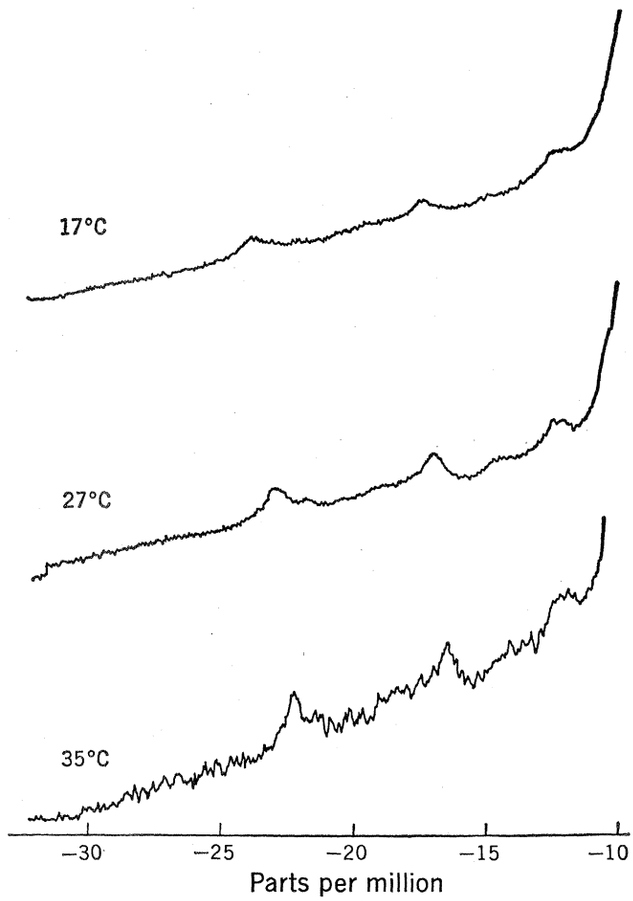


Fig. 1 (left). A comparison of the low-field NMR hyperfine shifted lines, at 22°C, of (top spectrum) deoxyhemoglobin and (middle and bottom spectra, respectively) the deoxy-α_{PMB}- and deoxy-β_{PMB}-chains. Fig. 2 (right). Low-field NMR spectra of deoxyhemoglobin at different temperatures. Most of the solvent H_2O has been replaced by heavy water (D_2O), and the deuterium ion concentration, expressed as pD, was adjusted to 8.0.

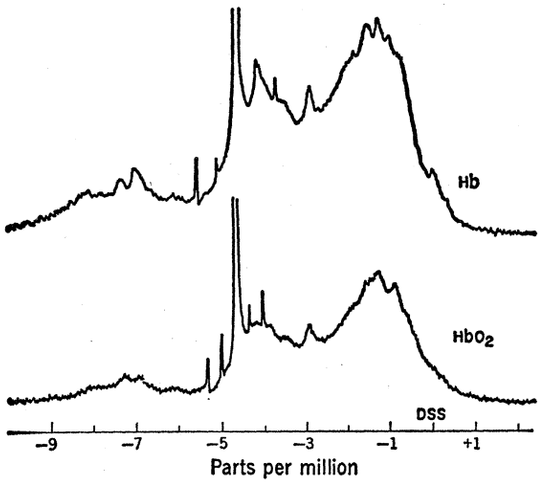


Fig. 3 (left). A comparison of the central region of the NMR spectra of deoxyhemoglobin with that of oxyhemoglobin. Temperature, 33°C; *p*D, 7.0.

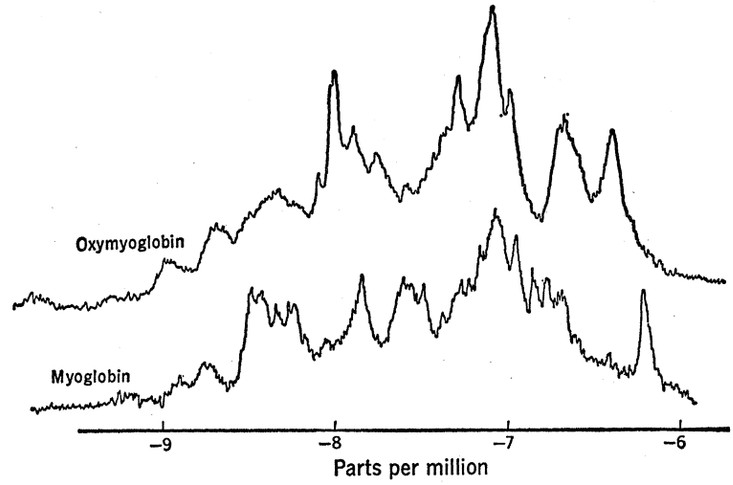


Fig. 4 (right). A comparison of the aromatic region of the NMR spectrum of deoxymyoglobin with that of oxymyoglobin.

An equivalent amount of oxyhemoglobin H was added to this oxidized α-chain preparation to give a mixed-state hemoglobin $[\alpha^{III}(H_2O)\,\beta^{II}(O_2)]_2$ (see *15*). Deoxygenation of samples was generally accomplished through alternating purified nitrogen gas with a vacuum, although, to prepare the deoxy- chains for NMR, dithionite was used. The state of oxygenation was monitored by means of optical absorption spectra. After deoxygenation, the NMR samples were stored at 4°C until used.

Mixed-state hemoglobin was also prepared from aged oxyhemoglobin by means of Bio-Rex 70 ion absorption chromatography (*16, 17*). After deoxygenation and after several hours at room temperature, the sample was oxygenated and rechromatographed. The chromatographic elution pattern was the same as before, indicating that negligible amounts of oxyhemoglobin A (HbO_2) or ferric hemoglobin A [Hb (H_2O)] tetramers were generated through heme, subunit, or electron exchange.

Experimental Results

The experiments are arranged in three sections. First, we compare the NMR spectra of the isolated hemoglobin chains with the spectrum of the tetramer; second, we present the NMR results which indicate conformational changes upon oxygenation; and third, we describe the resonance experiments on mixed-state hemoglobins.

Spectra of the chains and of the tetramer. Figure 1 shows the low-field region of the NMR spectra of deoxygenated α-chains and β-chains as their PMB derivatives and compares them with the spectrum of deoxyhemoglobin A (Hb). The spectra of the α-chains and the β-chains differ greatly from each other, and both differ greatly from the spectrum of the tetrameric form. The positions of these resonances are all temperature-dependent, and thus evidently arise from hyperfine interactions. For the case of Hb, the temperature dependence is shown in Fig. 2, where it may be seen that the resonance shifts decrease with increasing temperature, as one would expect for hyperfine shifts.

In addition to detecting these changes while the chains are in the deoxygenated state, it has been possible, by these magnetic resonance techniques, to detect differences between the isolated chains and the chains in the tetrameric form in ligated states. For example, EPR studies have shown that a frozen solution of $Hb(H_2O)$ in the high-spin state gives a single absorption in the vicinity of $g \approx 6$, with derivative extrema separated by ~ 40 gauss; this observation indicates that the iron environment has approximately tetragonal symmetry. Isolated ferric α-chains, $\alpha^{III}(H_2O)$ (see *17*), on the other hand, exhibit a split EPR signal with two well-resolved g-values of 6.18 and 5.78, both of which come from a single species in an environment of lower symmetry.

In an analogous way the shifted NMR resonances (*18*) of the cyanide complexes of the ferric α- and β-chains differ slightly from each other and differ slightly also from their positions in tetrameric ferric hemoglobin cyanide (HbCN) (*19*). These differences between the isolated chains and the tetrameric form are much smaller than those shown in Fig. 1, where the samples were in the deoxygenated state.

Conformational changes upon oxygenation. A comparison of the central region of the spectra of Hb and HbO_2 is presented in Fig. 3. In the spectrum of Hb, a well-resolved line with intensity corresponding to approximately six protons per subunit group is observed close to DSS. The position of this line was independent of temperature between 10° and 35°C. Hence this line is evidently shifted by ring currents and its position is sensitive to conformational changes in the molecule. The resonance observed at this position in Hb is not observed in HbO_2. The most likely explanation of its disappearance is that the subunits in HbO_2 and Hb have different tertiary structures.

Because the NMR spectra of the isolated chains and myoglobin are better resolved, it is easier to obtain details of tertiary structural changes upon oxygenation from these smaller proteins than from tetrameric hemoglobin. Evidence for conformational changes upon oxygen binding within the single chain of myoglobin has been obtained (*20*) by a comparison of the ring-current-shifted resonances in Mb and MbO_2. For example, one well-resolved temperature-independent resonance with the intensity of two protons is observed at -6.20 parts per million in Mb but not in MbO_2, as shown in Fig. 4. In the discussion below, reasons are given for associating this resonance with the two protons in the meta

positions of phenylalanine CD1. In addition to this particular resonance, there are many differences between the NMR spectrum of Mb and that of MbO_2 in the aromatic region shown in Fig. 4, as well as in other spectral regions—differences which appear to indicate extensive structural changes between the two states.

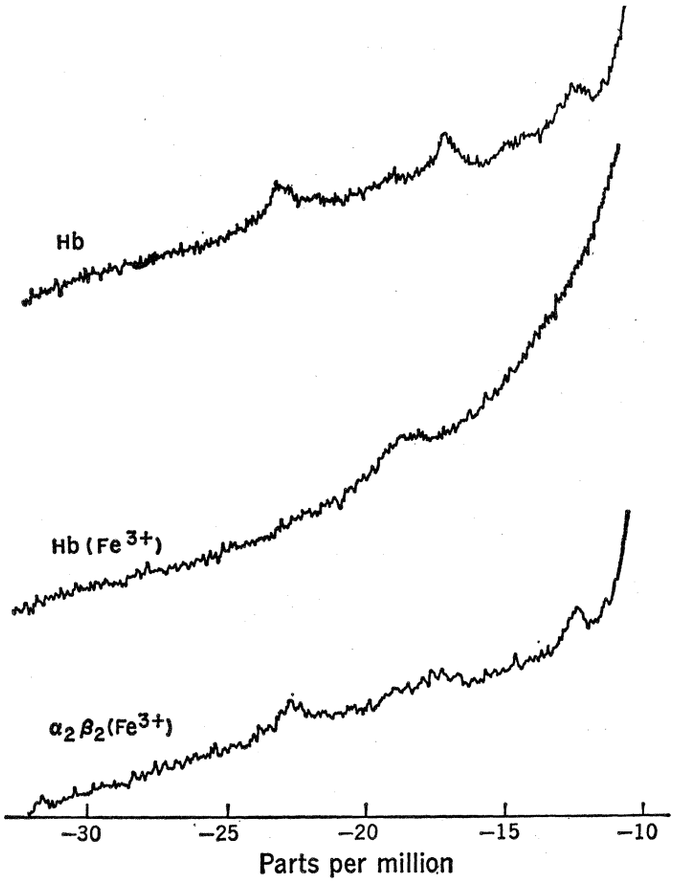
Mixed-state hemoglobin. Nuclear magnetic resonance experiments were made on both preparations of mixed-state hemoglobins described above, with similar results.

It has been reported elsewhere (*19*) that the hyperfine shifted resonances from the ferric heme cyanide in a mixed-state hemoglobin composed of oxy- and ferric cyanide chains were the same as those in ferric HbCN. This indicates that the distribution of unpaired electrons in the ligated heme of one chain was unchanged when the ligands of the other chains were changed from cyanide to oxygen. Recently we have determined that the shifted resonances in mixed-state hemoglobins composed of deoxy- and ferric chains (Fig. 5) and also deoxy- and ferric cyanide chains are a superposition of the resonances of Hb and $Hb(H_2O)$, and Hb and HbCN, respectively. Figure 5 shows that the shifted resonances at 25°C of deoxy- and ferric mixed-state hemoglobin are at the same positions as those observed for the corresponding tetramers, Hb and $Hb(H_2O)$, within the limits of experimental error. Similar results are obtained at 18°C, so evidently the temperature dependence of the resonances is the same as that of the homogeneous tetramers.

An analogous EPR experiment was performed at 1.4°K on the high-spin ferric heme groups in mixed-state hemoglobin composed of deoxy- and ferric chains prepared from aged hemoglobin (*14, 17*). The EPR signal of the ferric chains had a g-value of 5.92 ± 0.02 and a separation between derivative extrema of 41 ± 4 gauss in the oxygenated samples. These properties did not change as the ferrous chains in the mixed-state tetramers were alternately oxygenated and deoxygenated, and they are identical to the values observed in the ferric hemoglobin tetramers. In the deoxygenated form of $[\alpha^{III}(H_2O)\beta^{II}]_2$ which had been reconstituted from chains, a weak, broad line ~250 gauss wide appeared, in addition to this resonance. In thoroughly deoxygenated samples (~95 percent deoxygenated, as judged by optical spectroscopy) the broad line was present, with integrated intensities which ranged from 10 to 40 percent of the total intensity of the ferric chains. Because the weak broad line which appeared in the ferric α-chains when the β-chains were deoxygenated varied in intensity from sample to sample and was weakest in the sample that was shown, by chromatographic and optical criteria, to be the best, we regard it as due to an artifact of unknown origin.

Discussion

The NMR and EPR experiments on mixed-state hemoglobins show that the properties of the heme groups of the α-chains are not affected by the state of ligation of the β-chains in the same molecule, and vice versa. No differences were observed between the heme groups in mixed-state hemoglobins and in the corresponding homogeneous tetramers.



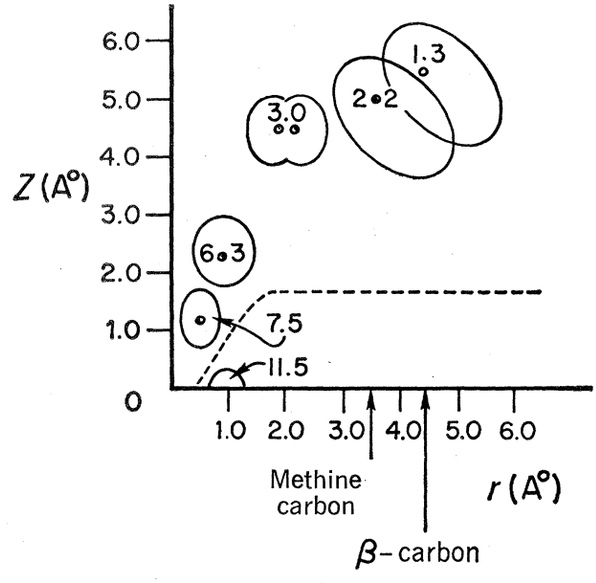


Fig. 5 (left). A comparison of the NMR spectra, at 220 megahertz, of (top spectrum) Hb^{II}, (middle spectrum) $Hb^{III}H_2O$, and (bottom spectrum) mixed-state hemoglobin $[\alpha^{II}\beta^{III}(H_2O)]_2$, showing that the mixed-state spectrum is a sum of the other two. Temperature, 25°C; *p*D, 7.0. Fig. 6 (above). A plot of the ring-current shifts measured (*28*) in porphyrin and phthalocyanine complexes where the positions can be deduced from the chemical formulas. The estimated uncertainties in the positions are indicated by the ellipses and are larger than the errors obtained in deriving the ring-current shifts from measured positions. The coordinates Z and r refer to the distances perpendicular to the plane of the ring and outward in the plane of the ring, respectively. The van der Waals thickness of the prophyrin π-electron system is indicated by the dashed line.

From the NMR spectra we have shown that the proton resonances of the hemes of the deoxy- chains in mixed-state hemoglobin composed of deoxy- and ferric chains are the same as in deoxyhemoglobin even though the former has a high affinity for oxygen (*21*), almost as high as that of the isolated chains, while deoxyhemoglobin has a low affinity for oxygen. Furthermore, the ligated ferric chains in the NMR experiments on mixed-state hemoglobins had the same NMR spectra regardless of whether the ferrous hemes in the other chains were or were not oxygenated. The ligated-chain spectra also remained the same when the other hemes were in the ferric states and were bound by the same ligand, either cyanide or water, to form homogeneously ligated tetramers.

In the parallel EPR experiment on the high-spin ferric chains in mixed-state hemoglobin, the major component of the low-temperature EPR spectrum was unchanged as the ferrous hemes were successively oxygenated and deoxygenated. Furthermore, this major component of the ferric-chain resonance in the mixed-state hemoglobin was the same as that observed in the ferric-hemoglobin tetramers. Results of this EPR experiment on mixed-state hemoglobin A differ from results of the experiment recently reported on Hb M Hyde Park (*22*), a hemoglobin in which tyrosine replaces the normal proximal histidine in residue $\beta 92$ and the β-chains are oxidized to the ferric state. In Hb M Hyde Park there was a change in the line-splitting in part of the EPR spectra of the ferric β-chains as the α-chains were alternately oxygenated and deoxygenated, but this dependence was not observed in two other M-type mutants. Thus, whereas in Hb M Hyde Park there is evidence for a change in the electronic properties of the ferric β-chain heme upon oxygenation of the α-chain heme, there is no such evidence in the case of the mixed states of normal hemoglobin.

In contrast to the lack of changes at the heme group of mixed-state hemoglobin during oxygenation, large differences between the properties of the heme groups of isolated chains and of tetramers are observed. In the deoxy-state these changes are expected, since it is well known that the Soret band of the optical spectrum changes with the change from isolated chains to tetramers (*23*). No changes in the optical spectra of isolated chains relative to tetramers when the hemes were in the ligated form have been reported. However, by EPR and NMR techniques we have been able to observe heme-group changes between chains and tetramers of the ligated ferric-state hemoglobins. These observations show the great sensitivity of these resonance techniques to the environment of the heme group.

The sensitivity of the NMR measurements to small conformational changes near the heme is further illustrated by an experiment on ferric myoglobin cyanide (MbCN). Changes of ~ 200 hertz in the position of a heme methyl group which we had tentatively identified as the methyl group on the right vinyl pyrrole are observed (*24*) upon the addition of cyclopropane to MbCN. X-ray crystallography has shown (*25*) that the cyclopropane sits above the right vinyl pyrrole, while the Fourier difference synthesis shows no change of heme crystallographic coordinates upon introduction of the cyclopropane. Hence, it is clear that NMR measurements of the heme-group protons are sensitive to changes which cannot be detected by x-ray crystallography. As a further illustration of the sensitivity of NMR line-shifts to the environment of the heme group, we note that the ferric cyanide forms of fetal hemoglobin (*26*) and the hemoglobins from various mammals (*27*) show NMR spectra different from the spectrum of the ferric cyanide form of human hemoglobin A.

The sensitivity of ring-current-shifted NMR lines to molecular structure is mentioned above, and Fig. 4 shows the difference between the resonances of Mb and MbO_2 in the aromatic region. In order to calibrate the dependence of ring-current-shifts produced by porphyrins (or porphyrin-like molecules), we have plotted in Fig. 6 values for these shifts reported elsewhere (*28*) relative to coordinates for compounds whose structure can be estimated reasonably well. The ellipses indicate uncertainties concerning the positions in the model compounds. Using Kendrew and Watson's coordinates for myoglobin (*29*), the known amino acid proton resonance positions, and an empirical expression for the porphyrin ring-current shifts designed to fit the measured shifts of Fig. 6, we have simulated the complete NMR spectrum of myoglobin. In this way we identified the resonance at -6.20 parts per million (shown in Fig. 4) with the two protons in the epsilon positions of phenylalanine CD1, since no other protons are expected within $\pm$ 0.4 part per million. In Fig. 4, it may be seen that in MbO_2 this resonance has moved downfield by at least 0.2 part per million. As may be seen in Fig. 6, this resonance shift corresponds to a displacement of $\sim$0.2 angstrom away from the iron in the likely event that the phenylalanine ring remains parallel to the porphyrin ring. Hence it is clear from these NMR studies that small but definite changes of structure have been determined within the myoglobin molecule when oxygen binds. As shown in Fig. 5, changes in ring-current-shifted resonances in hemoglobin are observed upon oxygenation, indicating that in hemoglobin, too, there are structural changes upon oxygen binding. It seems most likely that these changes in the hemoglobin NMR spectrum also come from changes of the tertiary structure.

At present it is not possible to calculate with any rigor how the NMR hyperfine shifts would respond to changing oxygen affinity of the heme group. However, our estimates indicate that these shifts should be approximately proportional to the binding energy, so it is hard to see how energy changes larger than a few percent could be undetected in the NMR measurements.

Yet ΔF_I is about 3 kilocalories per mole, out of a total (*30, 31*) oxygen-binding free energy of ~ 25 kilocalories per mole. Hence, if any appreciable fraction of ΔF_I had shown up at the other heme group as an enthalpy change, we certainly would have detected it in our measurements. Furthermore, if such a fraction had shown up at the heme as a conformation or entropy change—and Wyman's analysis of Roughton's data indicates that ΔF_I is indeed an entropy change (*2*)—then, again, it should have been detected because of the high sensitivity of these resonance measurements to structural changes near the heme.

In summary, NMR and EPR measurements are very sensitive to enthalpy changes and structural changes at the heme group, yet they do not show any changes in the different mixed-state hemoglobin samples. Therefore we conclude that ΔF_{IH} makes a negligible contribution to ΔF_I. In other words, the explanation of heme-heme interaction between heme groups separated by ~ 30 angstroms in hemoglobin is that there is none! While this conclusion is consistent with the idea that conformational changes are responsible for the cooperativity of ligand binding, it represents a departure from

previous thinking in that it definitely eliminates the heme group as the site of these free-energy changes. A negligible contribution by the heme group to ΔF_I helps to explain the well-known observation that ΔF_I is almost constant for many different ligands. Carbon monoxide (*32*), nitroso aromatics (*33*), various alkyl isocyanides (*34*), and oxygen all show only small variations in ΔF_I, despite the fact that they cover a wide range of heme-ligand binding energies.

In the absence of direct heme-heme interaction we must look for changes in the protein—that is, $\Delta F_I \approx \Delta F_{IP}$. The hypothesis which, from our experiments and the published results of others, seems most likely is the following. Ligand binding changes the conformation of the subunit to which the ligand binds. Our experiments show that these conformational changes are not transmitted to the heme group of the neighboring subunits. Experiments by others (*35*) have shown that ligand-induced conformational changes in the α-subunits are not strongly felt at the β93 residue of the β-subunits. Hence, ligand-induced conformational changes of the subunits cause the subunits to roll upon each other and to form the different quaternary structural arrangements revealed by the x-ray crystal-structure (*4*) determination. The free energy of cooperativity must be sought in protein itself, for example, in the different subunit interfaces of the ligated and unligated hemoglobin tetramers.

Koshland, Némethy, and Filmer (*36*) presented a general analysis of cooperativity in proteins composed of subunits. Haber and Koshland (*37*) subsequently extended these models of interacting subunits so as to include the symmetry-conserving case suggested by Monod, Wyman, and Changeux (*5*). Although their generalized models included many different physical mechanisms, the model which Koshland, Némethy, and Filmer actually used to fit the observed sigmoid oxygenation curves of hemoglobin was their so-called simplest sequential model in which ligand-induced changes were confined to one subunit and in which all the contributions to ΔF_I were made by interaction of the rigid subunits at their interfaces.

This model is consistent with our present results, which, however, do not rule out the possibility that ligand-induced changes are propagated a short distance across the interfaces, but not as far as to the next heme. While Koshland *et al.* fitted the oxygenation curves with their simplest sequential model, the fit could not be taken as proof of its validity. Monod *et al.* had previously shown that the same data could be explained by their allosteric symmetry-conserving model. Variants of Monod's tightly coupled model, such as Guidotti's dimer model (*38*), have also satisfactorily explained the oxygenation data.

Another limitation on the extent to which ligand-induced changes are transmitted across subunit interfaces comes from the spin-label experiments of Ogawa, McConnell, and Horwitz (*39*) on mixed-state hemoglobin. These experiments showed that the local protein conformation around the label at the β93 sulfhydryl group depends strongly on the state of oxygenation of the β-chain and less strongly on the states of oxygenation of neighboring α-chains. This means that there are ligand-induced conformational changes within a subunit, similar to those we have shown in myoglobin and hemoglobin NMR spectra, and that these conformational changes propagate to the region of subunit contact—that is, the α_1–β_2 contact, in this case, but that their effects beyond the interface are severely reduced.

Recent analogous experiments by Antonini and Brunori (*35*) show that the reactivity of the sulfhydryl group at β93 toward PMB depends only on the state of ligation of the β-chains, and therefore are also consistent with the model presented here.

Additional evidence for a ligand-induced conformational change is found in Hb M Hyde Park, in which part of the EPR of the ferric β-chain does change when the α-chain is oxygenated (*22*). As mentioned above, this change is not observed in our experiment on mixed-state hemoglobin or in other HbM samples, a fact which indicates that this mutant β-chain is more strongly influenced by oxygenation of the α-chain than the normal β-chain is. However, the interpretation of this experiment does require ligand-induced changes in the normal α-chain which can be transmitted to the $\alpha\beta$ interface region.

Our results show no evidence for the kind of conformational changes required by the allosteric model for hemoglobin proposed by Monod, Wyman, and Changeux (*5*). In the mixed-state hemoglobin molecules the deoxy- chains have high affinity, and in the model of Monod *et al.* the entire tetramer must be in its high-affinity form. But we have found no change in the vicinity of the unligated or ligated heme which could explain the high affinity. Hence, the differences in oxygen affinity must be looked for away from the heme—that is, in the somewhat remote parts of the protein, where they must exist as structural changes. But remote conformational changes in one subunit upon ligand binding could by themselves lead to subunit interactions, as we postulate above. One could postulate conformational changes which are large enough to account for the interaction but which are still remote from both the heme group and the subunit interface, so the experiments discussed here do not disprove this model. They do, however, narrow the range of possible allosteric mechanisms.

These results complement the x-ray structural determination because they suggest that the cooperativity is to be explained by changes at or near the interfaces between the subunits, which have already been shown, at a resolution of 5.0 angstroms, to be different for oxy- and deoxyhemoglobin and have been described, at a resolution of 2.8 angstroms, for oxyhemoglobin (*40*). When the structure of deoxyhemoglobin is obtained by Perutz and his collaborators at a resolution of 2.8 angstroms, that will be the time to ask just what specific ligand-induced conformational changes within the subunits might be responsible for the changes extending to the interfaces and to plan experiments to test these detailed mechanisms.

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Mousterian Cultures in European Russia

The Mousterian of European Russia comprised a series of cultures in a variety of environments.

Richard G. Klein

The term "Mousterian" refers to a complex of cultures which existed in Europe, in parts of central and western Asia, and in North Africa during the Last (or Eem) Interglacial and the first two-thirds of the Last (or Würm) Glacial. All Mousterian cultures were characterized by the manufacture of stone tools on flakes, including especially tool-types called sidescrapers, points, and denticulates.

In European Russia, the vast area of the Soviet Union west of the Urals and north of the Caucasus, Mousterian sites constitute the earliest well-documented evidence of human occupation. It is not yet clear whether the lack of undoubted pre-Mousterian sites means that Mousterian peoples were the first to settle the area or whether it reflects inadequacy of investigation (*1*). In any case, the available data suggest that the Mousterians succeeded in settling a large part of European Russia, including all the major river valleys up to at least the latitude of Bryansk (53.15°N) and quite possibly beyond.

Altogether, at least 33 Mousterian occupation sites have been uncovered in European Russia (Fig. 1), comprising ten open-air stations [Kasperovtsy (*2*; *3*, pp. 105–106), Molodova I and V (*3*, *4*), Khotylevo (*5*), Kodak (*6*, p. 22; *7*), Rozhok I and II (*8*), Nosovo I (*8*), Volgograd (*9*), and Il'skaya (*10*)], and 23 cave sites [in the Prut Basin, Buteshty and Starye Duruitory (*11*); in the Dnestr Basin, Vykhvatintsy (*12*); on the Northern Black Sea Littoral, Il'inka (?) (*6*, pp. 19–20; *13*); in the Crimea, Kiik-Koba (*14*), Starosel'e (*15*), Shajtan-Koba (*16*), Chokurcha (*17*), Kabazi (*18*), Volchij Grot (*19*), Adzhi-Koba (*20*), Kholodnaya balka (*21*), Bakhchisarajskaya (*22*), Chargorak-Koba (*23*), and Kosh-Koba (*24*); in the Northern-Caucasus Foreland, Dakhovskaya Cave (*25*) and Gubs Cave and Shelter No. 1 (*26*); on the Eastern Black Sea Littoral, Akhshtyr' Cave (*27*), Navalishino Cave (*28*), Vorontsovo Cave (*29*), Khosta Caves I and II (*30*), Ats Cave (*29*)].

Geological Age of the Sites

Only the open-air sites may be placed in time on the basis of stratigraphic evidence alone. A provisional chronological ordering of them is presented in Fig. 2 (last column). The single candidate for a Last Interglacial site is Khotylevo, near Bryansk, where the lone occupation horizon occurs more than 20 meters from the surface in what is probably Last Interglacial alluvium. Khotylevo is located considerably farther north than any of the other known Mousterian sites—a fact which may well be linked with its Last Interglacial age. The next youngest sites are probably Il'skaya (lower level) and Volgograd. In each case, occupational debris was found on the surface of a thick fossil soil believed to have developed during the Last Interglacial. The preservation of cultural materials undisturbed on the surface of the soil indicates that they were sealed in soon after the site was abandoned. The colluviation which accomplished this probably began at, or shortly after, the onset of the Last Glacial. Thus, in both cases, the cultural horizons can be no older than the terminal part of the Last Interglacial and may well be of earliest Last Glacial age.

The remaining open-air stations, like the majority of Mousterian sites elsewhere, date rather clearly from the earlier part of the Last Glacial. At Nosovo I and Il'skaya (upper level), the occupation horizons occur only a short distance above deposits believed to be of Last Interglacial age. An early-to-middle Last Glacial age for the five Mousterian levels of Molodova I and

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