Visible Absorption and Electron Spin Resonance Spectra of the Isolated Chains of Human Hemoglobin. Discussion of Chain-Mediated Heme–Heme Interaction*

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ABSTRACT: The optical absorption and electron spin resonance spectra of the isolated human hemoglobin chains in the ferri state have been studied. The spectra of the isolated α^+ chains are very similar to those of ferrihemoglobin, but those of the β^+ chains are significantly different. The atypical spectra of the β^+ chains are shown to arise from an altered spin equilibrium of the heme iron. At 0°, the aquo derivative of β^+ is about 35% low spin; at liquid nitrogen temperature, the same compound is seen from electron spin resonance spectra to be 67% low spin compared with only about 5% for α^+ and Hb^+. The spin equilibrium of the β^+ chains is thus greatly modified on going over to native $Hb^+(\alpha_2^+\beta_2^+)$ through combination with the α^+ chains. This shift is interpreted as arising from a change in the interaction between iron and histidine, resulting in a slight lengthening of the Fe–N bond. On the other hand, a similar shift in the spin equilibrium of the β^+ chain is shown to occur on modifying the free thiol group next to the heme-binding histidine. Conformational strains on the peptide chain close to the heme can thus alter the spin equilibrium of the heme iron. In the reduced deoxygenated form, the β chains have a Soret band maximum at a wavelength shorter than that for α chain or ferrohemoglobin. Association with the α chain thus results in a shift of the maximum to a longer wavelength. A similar shift is observed on modifying the thiol next to the heme in the β chain. The change to longer wavelengths is in both cases associated with diminished oxygen affinity. The possibility that the Soret band shifts result from altered spin equilibrium of Fe(II) is considered. The results are discussed in the context of heme–heme interactions in native hemoglobin.

The optical absorption of the heme chromophore has been correctly looked upon as a sensitive probe of heme–protein interactions in hemoglobin. The absorption spectra of the different derivatives (free or gas-liganded ferrous, liganded ferric–H_2O, OH^-, CN^-, F^-, etc.) have therefore been frequently studied. However, since the mammalian hemoglobins are known to contain two distinct types of chains (a pair of α and β in normal human Hb), additional information to that on the whole protein is required in order to appreciate the factors which give rise to the properties of each type of chain and their mutual interactions in hemoglobin. In fact, some of the remarkable properties of hemoglobin, namely, heme–heme interaction (cooperative ligand binding) and ligand-linked ionizations (Bohr effect), are now generally believed to generate from specific interactions between chains, particularly the unlike ones.

A method for the preparation of the isolated chains (α and β) of human hemoglobin was reported in 1965 by Bucci and Fronticelli; it is thus pertinent to attempt an analysis of the total spectrum of the tetramer ($\alpha_2\beta_2$) in terms of that of the chains. A study along these lines has been made by Antonini *et al.* (1965) and Brunori *et al.* (1968) on the deoxy forms. A similar approach could not be made to the study of the oxidized forms, as the ferri chains are extremely unstable in aqueous solution. A method for their preparation in a relatively stable form is now available and has been reported elsewhere (Banerjee and Cassoly, 1969). In the present study, we shall describe the visible absorption spectra of the α^+ and β^+ chains of human hemoglobin, both in the aquo and hydroxide forms. Deductions from the spectra as to the spin states of the iron in these proteins will be corroborated by electron spin resonance spectroscopy. Some new results on the ferrous unliganded forms of the separated chains as well as of hemoglobin will also be reported and discussed.

Experimental Section

Once crystallized human hemoglobin was obtained from fresh hemolysates by the use of 2.8 M phosphate buffer according to the method of Drabkin (1946). Methemoglobin was obtained by adding the equivalent amount of potassium ferricyanide to deoxyhemoglobin in pH 7 phosphate buffer under vacuum in a Thunberg tube; the product was dialyzed against dilute NaCl (2×10^{-3} M).

Hb_{PMB}[1] was obtained by adding 2 equiv of PMB to oxy- or carboxyhemoglobin (four chains) at pH 7. The saturation of free thiol groups by the PMB was always verified either by

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[1] Abbreviations used: Hb, ferrohemoglobin; Hb^+, ferrihemoglobin; α_{PMB}, β_{PMB}, chains with thiol groups transformed into *p*-mercuribenzoate (PMB); α_{SH}, β_{SH}, chains with free thiol groups; α_{SH}, β_{SH}, etc., ferro chains; α_{SH}^+, β_{SH}^+, etc., ferri chains. The α_{PMB} and β_{PMB} chains described here have all the thiol groups bound to mercury but in Hb_{PMB} only the two cysteines close to the heme in the β chains are modified. In making comparisons between the spectra of the different forms we shall refer to the α_{SH}, α_{PMB}, β_{SH}, β_{PMB}, Hb, and Hb_{PMB} forms of the protein although the β_{SH} and β_{PMB} chains and Hb and Hb_{PMB} are associated.

polarographic titration (Cecil and Snow, 1962) or by the spectrophotometric method of Boyer (1954). The results obtained by the two methods agreed closely.

α_{PMB} and β_{PMB} were obtained in the CO-liganded form by the method of Bucci and Fronticelli (1965). The regeneration of free thiol groups was achieved for α chains by dialysis against thioglycolate as described by the same authors, and for the β chains by the use of dodecanethiol (De Renzo *et al.*, 1967). The number of free thiol groups recovered was 0.95–1 in the case of α chains and 1.8 ± 0.1 (per heme) in the case of β chains.

The ferri chains were prepared by the action of equivalent amounts of potassium ferricyanide on the deoxy derivatives dissolved in 1 M (or sometimes 1.5 M) glycine as described elsewhere (Banerjee and Cassoly, 1969). The stability of these materials is greatly enhanced by chilling to 0° immediately after oxidation. The chains were examined shortly after preparation, in the same solvent (1 or 1.5 M glycine) at a temperature close to 0°. The concentrations of all the hemoproteins described here were determined by spectrophotometry as the cyanomet derivative in 1 M glycine (ϵ_{mM} at 540 mμ = 11.5/heme).

The absorption spectra were measured using a Cary 14 recording spectrophotometer equipped with a special thermostatic attachment for work near 0°.

Electron Spin Resonance. The electron spin resonance spectra were measured using a Varian E 3 spectrometer. The sample was placed immediately after preparation in a 3-mm wide quartz tube and frozen to 77°K in a Varian V 4546 Dewar adapted to the spectrometer cavity. A current of dry nitrogen was allowed to flow continually into the quartz sample container in order to prevent the condensation of atmospheric oxygen.

The microwave power and modulation (100 KHz) were maintained, respectively, at 20 mW and 5 G for all the experiments.

After the study of each sample spectrum, a blank was run with the solvent (1.5 M glycine–0.05 M phosphate, pH 5.9 at 20°) under identical conditions. The net spectrum was obtained by subtraction. The $g = 2$ region was recorded once again with a fivefold higher gain and corrected against the blank spectrum taken with the same gain. The proportion of high- and low-spin forms was obtained by double integration of peaks in the $g = 6$ region and those in the $g = 2$ region.

Results

Visible Absorption. FERRI SPECTRA. Figure 1a,b shows the visible absorption spectra of α_{SH}^+ and β_{SH}^+ in 1 M glycine (pH 5.9 and 9.5) at 1°. It will be seen that the spectra of α_{SH}^+ both in the conjugated acid and hydroxide forms are very similar to those of methemoglobin (dotted lines). On the other hand, the acid spectrum of β_{SH}^+ is clearly different, and shows the contribution of two additional bands at about 535 and 570 mμ, respectively; the increased absorption (compared to methemoglobin) in these regions is compensated by a decrease at 500 and 630 mμ. The two extra bands are not due to the presence of residual unoxidized oxy chains, since they persist when excess ferricyanide is used. The spectra were reproducible in numerous independent preparations. Moreover, no significant time-dependent changes were noticed over several hours after preparation.

The spectra of α_{PMB}^+ and β_{PMB}^+ were less reproducible as

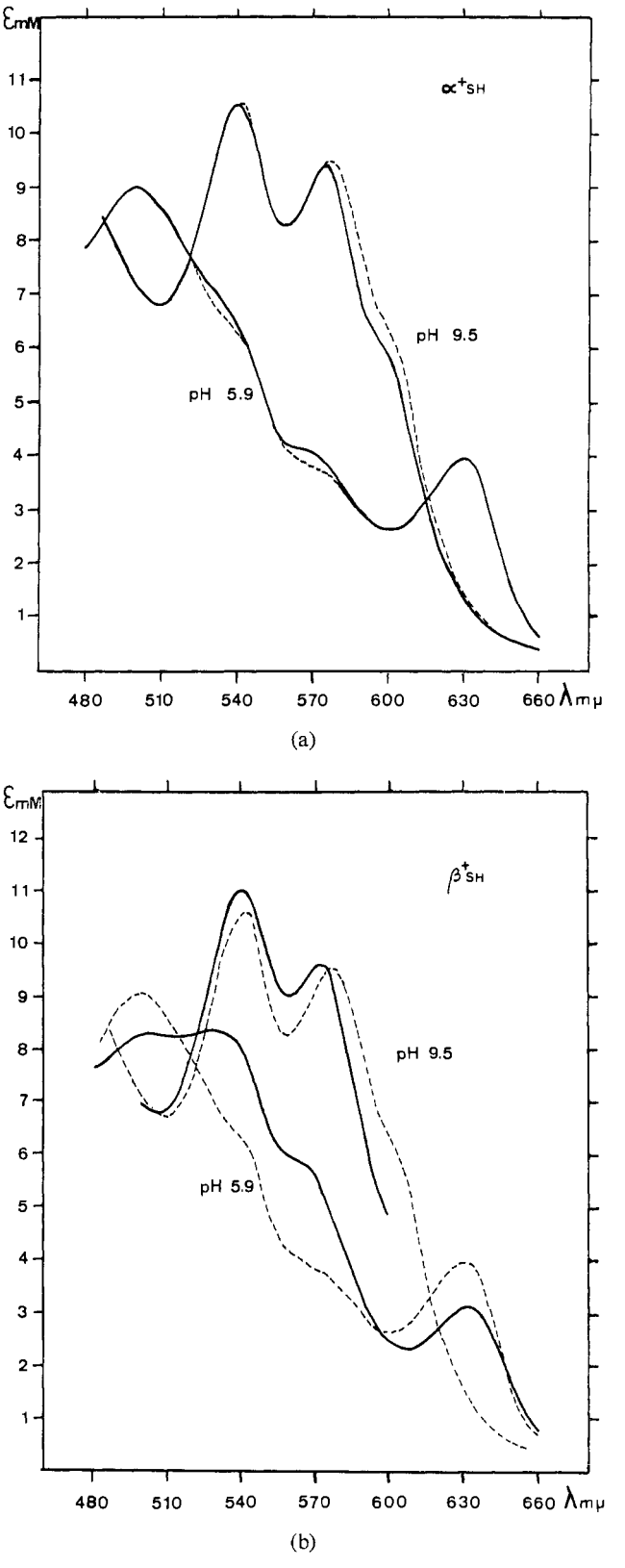

FIGURE 1: Visible absorption spectra (a, left and b, right) of the ferri chains (α_{SH}^+ and β_{SH}^+ in 1 M glycine (pH 5.9 and 9.5) at 1° (solid curves). Absorption spectrum of methemoglobin under identical conditions is represented by the dotted curve, in wavelength regions where it does not coincide with the solid curve. Concentrations were normalized for the chains and for methemoglobin by spectrophotometry on the cyanomet derivatives prepared in 1 M glycine (ϵ at 540 mμ taken to be 11.5×10^3 for each).

TABLE I: Visible Absorption Spectra of the Ferrous Free, Oxy, and Carboxy Derivatives of Human Hemoglobin and Hemoglobin Chains.[a]

Hemoprotein	Derivative	Max (mμ)	Min (mμ)
Hb	Free	555 (13.3)	
	Oxy	576 (15.15); 542 (14.25)	560 (8.7)
	Carboxy	568.5 (14.95); 538.5 (14.85)	555 (11.9)
α_{SH}	Free	557 (13.8)	
	Oxy	576 (15.05); 541 (14.4)	560 (8.5)
	Carboxy	568 (14.35); 537 (14.1)	553 (11.2)
α_{PMB}	Free	557 (14.1)	
	Oxy	576 (15.4); 541 (14.2)	560 (8.4)
	Carboxy	568 (15.15); 537.5 (14.2)	553.5 (11.8)
β_{SH}	Free	558 (13.2)	
	Oxy	577 (14.5); 542 (13.9)	561 (8.85)
	Carboxy	569.5 (14.4); 540 (14.2)	555 (10.8)
β_{PMB}	Free	558 (13.9)	
	Oxy	577 (15.1); 542.5 (14.05)	561 (9.1)
	Carboxy	569.5 (14.7); 540 (14.55)	555 (12.0)

[a] Figures within brackets represent the millimolar extinction coefficient at the given wavelengths. The positions of the wavelength maxima or minima are believed to be accurate to better than 5 Å. The errors in extinction coefficients are less than 5%.

a slow change was observed with time (significant over 30 min), even at 1°. The rate of the transformation was somewhat different with different chain preparations. However, experiments on a large number of preparations show that the *initial* spectrum of α_{PMB}^+ is very close to that of α_{SH}^+; on the other hand, the initial spectrum of β_{PMB}^+ is about half way between those of β_{SH}^+ and Hb^+.

Figure 2 illustrates a typical recombination experiment, where equivalent amounts of α_{SH}^+ and β_{SH}^+ were mixed. The spectrum of the resulting solution is that of methemoglobin.

TABLE II: Spin-State Composition of Hemoproteins.[a]

Hemoprotein	Aquo Form		Hydroxide Form
	From Optical Absorption Spectra	From Electron Spin Resonance Spectra	From Optical Absorption Spectra
α_{SH}^+	~95	96	15
α_{PMB}^+	~95	95	
β_{SH}^+	~65	33[b]	5
β_{PMB}^+	~95	95	
Hb^+	~95	91	
Hb_{PMB}^+	~90	92	

[a] The figures indicate the proportion (in per cent) of high spin to total. [b] The differences between the results for the per cent high spin using the two different methods is within that to be expected from the temperature difference for the two types of measurements.

The fact that native methemoglobin was formed was corroborated by the stability of the product when glycine was washed out by dialysis, by electrophoretic analysis, and by oxygen binding behavior of hemoglobin obtained therefrom (Banerjee and Cassoly, 1969). These results show that the atypical spectrum of β_{SH}^+ is not due to the presence of an irreversibly denatured protein in the commonly employed sense.

Effect of Temperature. As has been stated above, the ferri chains are stable in concentrated glycine solution at a temperature near zero. The relative instability of the material at higher temperatures (~20°) did not permit a thorough analysis of the effect of temperature on the spectra. Nevertheless some qualitative information has been obtained. Raising the temperature (from 1° to 15°) has negligible effect on the spectrum of α_{SH}^+; on the other hand, the effect on β_{SH}^+ is appreciable. At 15° the bands at 535 and 570 mμ diminished in height and the bands at 500 and 630 mμ increased, so that the spectrum was nearer to that of Hb^+.

FERRO SPECTRA. Small but significant differences have been observed between the visible absorption spectra of a given derivative (deoxy, oxy, and carboxy) of the two types of chains and of native hemoglobin. The results are summarized in Table I. The concentrations of all samples were determined as the cyanomet derivative in 1 M glycine (ϵ_{mM} 11.5/heme at 540 mμ) and the extinction coefficients were calculated on this basis. The procedure might seem arbitrary, since the extinction coefficient of the cyanomet derivative may well be different for the different hemoproteins. However, Brunori *et al.* (1968) have recently reported a kinetic difference spectrum between the summed reactants and the reaction product of an equimolecular mixture of deoxygenated α_{SH} and β_{SH} chains (*i.e.*, deoxyhemoglobin); these results are relatively free from ambiguity arising from extinction coefficients chosen for the estimation of chains, since the concentrations (uncombined and combined) remain the same in the reaction cell throughout the

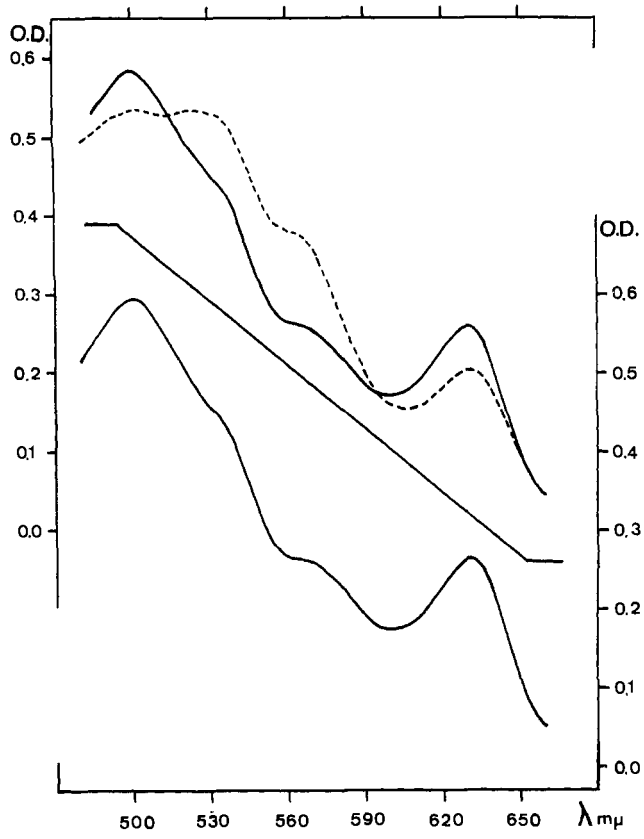

FIGURE 2: Illustrating a recombination experiment in which α^{+}_{SH} and $\beta_{SH}{}^{+}$ at pH 5.9 in 1 M glycine, at 1°, and having spectra given in the top panel, were mixed in equal proportions. The spectrum of the mixture (bottom panel) is that of methemoglobin, and did not change when glycine was washed out by dialysis. Concentration: 6.4 $\times 10^{-5}$ M for both; 10 mm light path. Left ordinates (optical density) correspond to the top panel, right ordinates to the lower one.

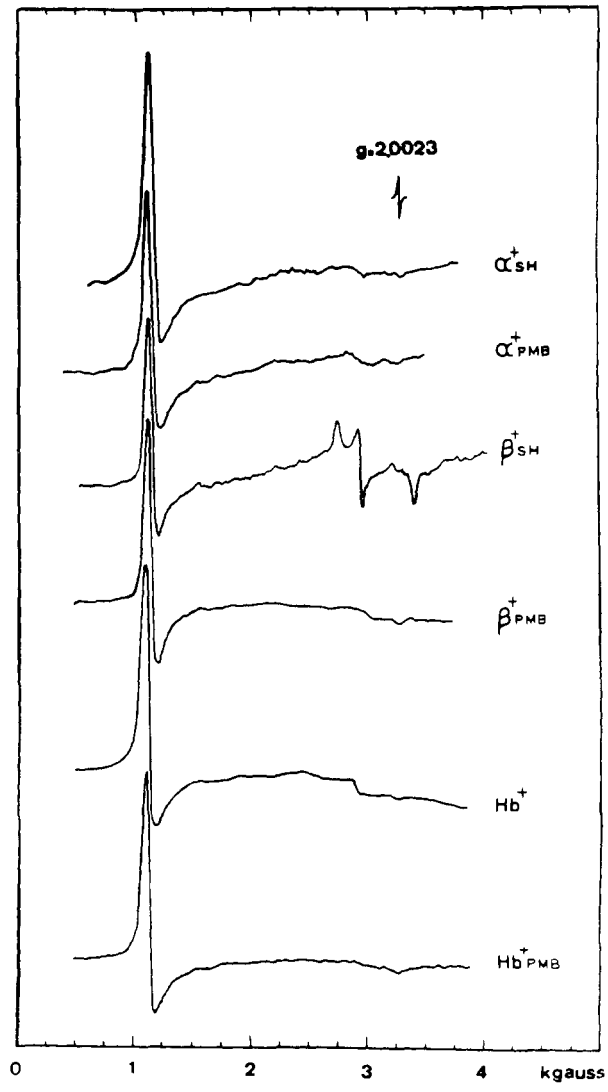

FIGURE 3: Electron spin resonance spectra of $\alpha_{SH}{}^{+}$, $\alpha_{PMB}{}^{+}$, $\beta_{SH}{}^{+}$, $\beta_{PMB}{}^{+}$, Hb^{+}, and $Hb_{PBM}{}^{+}$ in 1.5 M glycine–0.05 M phosphate (pH 5.9) (at 20°); temperature 77°K. $g = 2.0023$ corresponds to that of 1,1-diphenyl-2-picrylhydrazyl.

course of the experiment. We have constructed a similar difference spectrum from our static spectra of α_{SH}, β_{SH}, and Hb; the result is very similar in trend with that reported by Brunori *et al.*, a finding which suggests that the estimation of hemoglobin chains as well as the parent molecule by the use of a single extinction coefficient of the cyanomet derivative may be a reasonably good and rapid way to proceed. The statements made in Table I about the position of the bands are, of course, free from the restrictions imposed by the estimation procedure.

Electron Spin Resonance Spectra. Figure 3 shows typical electron spin resonance spectra obtained with the conjugated acid forms of $\alpha_{SH}{}^{+}$, $\alpha_{PMB}{}^{+}$, $\beta_{SH}{}^{+}$, $\beta_{PMB}{}^{+}$, Hb^{+}, and $Hb_{PMB}{}^{+}$. A detailed analysis of these spectra as well as of those obtained with the hydroxide, fluoride, and azide derivatives will be published elsewhere (Y. Henry and R. Banerjee, in preparation). It is clear from Figure 3 that the aquo derivatives of all compounds studied except $\beta_{SH}{}^{+}$ are essentially high spin. The notable exception, $\beta_{SH}{}^{+}$, shows a considerable amount of the absorption of the low-spin ($S = 1/2$) type. The high-field spectrum shows three structures with g values of 1.92, 2.23, and 2.39. For our present purpose, we report in Table II the calculated proportions of high- and low-spin types in the different hemoproteins studied. The difference in electron spin resonance spectra between $\beta_{SH}{}^{+}$ and $\beta_{PMB}{}^{+}$ is indeed specifically linked to the state of the thiol groups (free or mercury bound), and not due to secondary effects. This was shown by experiments on a sample of β_{PMB}, on β_{SH} derived by removal of Hg from β_{PMB}, and finally on β_{PMB} obtained by the fixation of mercury on the same β_{SH}. The initial proportion of high-spin component of β_{PMB} was restored when mercury was refixed on the free thiols.

Discussion

In many heme proteins it has been observed that the high- and low-spin forms of iron(III) exist in equilibrium. We may estimate the percentage of the two different forms directly from the visible absorption spectrum. We have made such estimates, applying the method of Smith and Williams (1968) for the derivatives described in the present paper. The characteristics of the high- and low-spin forms of typical aquo and hydroxide derivatives of Fe(III) myoglobin as calculated from the mixed states by Smith and Williams were taken as reference points (Table III). The results are shown in Table II

TABLE III: Spectra of Fe(III) Aquo and Hydroxide Complexes of Myoglobin.[a]

	High Spin	Low Spin
Aquo	502 (10.2); 630 (4.0)	535 (12.0); 570 (~5.0)
Hydroxide	490 (~10.0); 595 (~10.0)	540 (12.0); 575 (~10.0)

[a] Figures indicate λ_{max} and (in parentheses) ϵ_{mM}.

which also includes the figures obtained by electron spin resonance spectroscopy on the aquo forms. From the table it is seen that modification of the cysteine in the α chain has little effect on the spin equilibrium but that modification of the cysteines in the β chain drastically reduces the amount of low-spin form. Combination of the α and β chains similarly reduces the low-spin component in the β chains while modification of the two β-chain cysteines in the native $\alpha_2\beta_2$ unit possibly increases slightly the amount of the low-spin form. The agreement between the results obtained by visible spectrophotometry and by electron spin resonance spectroscopy are indeed very satisfactory. One is tempted to interpret the data in the light of what is known on the structure of these derivatives.

The two chains, α and β, of hemoglobin[2] differ in amino acid composition. The important distinction from the point of view of this paper is that in the α chain the single cysteine residue, 104, is a considerable distance both in sequence and in space from the heme unit while one of the two cysteines in the β chain, (F9) 93, is adjacent to histidine (F8) 92. This histidine binds directly to the iron of heme. Moreover the cysteine is in the short chain of amino acids which link this histidine with the contact region between the α_1 and β_2 chains in the native Hb (Perutz *et al.*, 1968).

In an analysis of the spectra of the derivatives of myoglobin, hemoglobin, peroxidase, and catalase, Brill and Williams (1961) showed that a change from high toward low spin was brought about by increasing the coordinating power of the ligands bound to the iron. For example, a change from water to hydroxide brings about a considerable swing in the spin-state equilibrium. Now the differences in spin-state equilibrium observed for the compounds studied in this paper are not due to a change in the ligands binding the iron, for (a) no coordinating ligands are introduced into the solutions and (b) a change from water to hydroxide has been prevented by working at pH 5.9 which is remote from the pK_a of all the derivatives, all close to 8 (Banerjee and Cassoly, 1969). We therefore consider that the changes in spin state described in Table II are brought about by a change in the interaction between the iron and histidine. In particular this interaction must be different in the β_{SH} as opposed to other chains. Such a change is almost a necessity on modifying cysteine 93 in the same chain but is also likely on going from the pure β_{SH} form to the native Hb ($\alpha_2\beta_2$) for this introduces strong interaction between the α and β chains at the amino acids 97–102 in the β chains (Table II of Perutz *et al.*, 1968). A combination of α and β chains apparently weakens the Fe–histidine bonds in the β chains.

A quite separate experimental indication in support of this view is available from redox potential measurements. As has been pointed out (Williams, 1968), a lengthening of the ligand nitrogen to iron bond by steric factors in a protein should result in an increase in redox potential for this change will not affect the stability of the ferrous as much as it will affect the stability of the ferric state. On going from the separate α chains, redox potential of +0.05 V, and β chains, redox potential +0.11 V, to native Hb, redox potential +0.17 V, at pH 6, just such an increase in the potential is observed (Banerjee and Cassoly, 1969). From this study of the ferric forms we conclude that a major effect of chemical or conformational alterations in the peptide sequence from 93 to 102 (β chains) is to alter the Fe–nitrogen (histidine 92) distance. Concomitant with the alteration in Fe–histidine distance, there must be small changes in the interaction between the iron and the porphyrin, but as yet we do not have experimental evidence thereof. It is of great importance to consider how a change in Fe–histidine distance could influence the Fe(II) complexes.

Ferrous Forms. There has been some previous work (Antonini *et al.*, 1965) on the spectra of the isolated chains in the reduced form. More recently, difference spectra between α and β chains (summed) and Hb (native) have been reported (Brunori *et al.*, 1968). In detail these authors observed that Hb absorbs more strongly than the sum of the separated chains in the Soret band region and also at certain longer wavelengths. They also report that a number of other modifications in the protein can induce heme spectral changes. These intensity changes are typical of the broadening of aromatic spectra in general on increase of temperature and are associated with an increase in the vibrational lability of the molecules.

While we have been able to confirm all the above spectroscopic features by direct study of the absolute spectra of the separated chains and their modified forms listed earlier, our study allows a more detailed inspection of the spectra themselves (Tables I and IV). Particularly interesting are the positions of the Soret maxima of the deoxy derivatives (Table IV). Both α_{SH} and α_{PMB} have their maxima at 4300 Å. In contrast, that of β_{SH} is at a wavelength (4285 Å) significantly shorter compared to that of β_{PMB} (4310 Å). Moreover, the Soret maximum of Hb (4305 Å) is at a longer wavelength than would be expected from the summed α_{SH} and β_{SH} chains. Previous work has shown that such shifts of the Soret bands can arise from changes in the spin state of the Fe(II) proteins (Williams, 1961). While at this stage we cannot give a unique explanation

[2] In the discussion we shall refer to the structure of horse hemoglobin although all the work done in the paper was performed on the human protein. Perutz and Lehmann (1968) faced with a similar comparison state that the difference in origin of the two proteins should not matter because the tertiary and quaternary structure of human and horse hemoglobin are indistinguishable at 5.5-Å resolution and because most harmful replacements affect residues which are common to all mammalian hemoglobins of known sequence.

for the observed shifts, this previous work and the above study of the Fe(III) proteins indicate the following possibility. On modifying the β_{SH} chain (with PMB), or on combining it with the α_{SH} chain, small alterations could occur in the Fe–histidine bond length. Such changes would obviously alter the Fe(II) spin-state equilibrium and thus explain the shifts in the Soret band. Moreover, the changes in the iron binding would be expected to alter the oxygen affinity of the chains. This is what occurs in fact, since β_{SH} has a higher oxygen affinity than β_{PMB} or Hb (Antonini *et al.*, 1965). The increase in oxygen affinity occurs with a Soret band shift to shorter wavelength. In a general sense a reason can be given for this situation. Other things being equal a major energy which militates against the uptake of oxygen by hemoglobin is the spin-pairing energy, *i.e.*, the energy required to take Fe(II) from high to low spin. Thus the nearer the Fe(II) can be brought to the crossover condition between the spin states, by the coordination of the protein, the better will be the binding energy for oxygen.

Although the spectra of the oxy and carbon monoxy complexes of the different hemoglobins show some small differences between the different proteins (Tables I and IV), we have not been able to attach significance to these changes as yet. We stress that all these derivatives are virtually 100% low spin and therefore the extremely sensitive probe of the protein, the spin-state balance, has been lost.

In conclusion, the spectroscopic evidence presented here is consistent with the following remarks on heme–heme interaction in hemoglobin. We start from the demonstrated importance of the $\alpha_1\beta_2$ chain interaction in the structure of hemoglobin (Perutz *et al.*, 1968). Bonding of one of the iron atoms to oxygen will alter its Fe–N distance and this alteration will be transmitted through the relevant peptide chain to the overlap region between α and β chains and then relayed to the next heme through an adjustment first in the peptide of the second chain and then in its iron–histidine distance (heme–heme interaction). It is then a function of the interchain interactions in the contact regions to maintain the iron–histidine distances in a condition of lability, so that even very small changes, *i.e.*, pH, ionic strength, etc., can change this distance and consequently oxygen binding properties. All these distance changes could be less than 0.2 Å in magnitude, and it is difficult to see how they can be detected except by spectroscopy.

TABLE IV: Position of the Maximum of the Soret Band for the Ferrous Free and Carboxy Human Hemoglobin and Hemoglobin Chains.[a]

Hemoprotein	Derivative	Max (mμ)
Hb	Free	430.5 (145)
	Carboxy	419.5 (208)
α_{SH}	Free	430 (120)
	Carboxy	418.5 (220)
α_{PMB}	Free	430 (120)
	Carboxy	418.5 (215)
β_{SH}	Free	428.5 (117)
	Carboxy	420 (201)
β_{PMB}	Free	431 (120)
	Carboxy	420 (210)

[a] Figures within brackets represent the millimolar extinction coefficient at the given wavelengths. The extinction coefficients are believed to have an error less than 5%. The error in wavelength (max) does not exceed 5 Å.

Added in Proof

After the present paper had been submitted Shiga *et al.* (1969) showed that slight differences between α and β chains could be seen in the electron spin resonance spectra of the respective nitroso complexes. These differences, though concerning the nitroso groups fixed on divalent iron, are in agreement with our results on the intrinsic nonequivalence of the α- and β-chain hemes.

The g values (1.92, 2.23, and 2.39) observed by us in the electron spin resonance spectra of β_{SH}^+ are very close to those reported for denatured methemoglobin (Hollocher, 1966; Hollocher and Buckley, 1966). These bands have been interpreted by the above authors as being due to the irreversible formation of a hemichrome. We have shown in the present work that the low-spin bands of the β_{SH}^+ spectrum disappeared on association with α_{SH}^+ or on treating the free thiol group with PMB. Whether or not this low-spin component is equivalent to a hemichrome is being investigated in detail and will be reported shortly.

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