The pH dependence of naturally occurring low-spin forms of methaemoglobin and metmyoglobin: an EPR study

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The paramagnetic species in human metHb and horse metmyoglobin (metMb) have been studied at low temperature using EPR spectroscopy. The high-spin (HS) haem signal in aquometMb has a greater rhombic distortion than the HS metHb signal. Nevertheless, the individual line width ($g = 6$) is smaller in metMb than in metHb, consistent with non-identical signals from the α and β Hb subunits. Three low-spin (LS) haem forms are present in metHb, while metMb has only two. The major LS form in both proteins is the alkaline species (with OH^- at the sixth co-ordination position). The minor LS forms are assigned to different histidine hemichromes in equilibrium with the normal HS species at low temperature. LS forms disappear when the haem is bound by a ligand, such as fluoride, which ensures 100% occupancy of the HS state both at room temperature and at 25 K. The small differences in effective g -factors of the histidine

hemichromes are interpreted in terms of different distances between the distal histidine and haem iron. The pH dependence of the inter-conversion of the different paramagnetic species is consistent with a model whereby protonation of a residue with a pK of 5.69 (metHb) or 6.12 (metMb), affects ligand binding and transformation from the HS to the LS form. Chemical and spectroscopic considerations suggest that the residue is unlikely to be the proximal or distal histidine. We therefore propose a model where protonation of this distant amino acid causes a conformational change at the iron site. Identical effects are seen in frozen human blood, suggesting that this effect may have physiological significance.

Key words: hemichromes, histidine, human blood, mechanism simulation.

INTRODUCTION

Mammalian Hb consists of two α and two β subunits containing 141 and 146 amino acids respectively (human Hb), while myoglobin (Mb) is monomeric with 153 amino acids in the horse protein [1]. Each subunit contains one haem group, with an essentially similar haem environment in all three types of monomer. In the ferric ('met') state, the haem iron in both proteins is largely high-spin (HS) in character at room temperature and neutral pH. At alkaline pH both haemoproteins become low-spin (LS), with a pK of approx. 8 for metHb and a pK of approx. 9 for metMb [2]. MetHb can also exist in two conformational states, 'R' and 'T', the equilibrium between which is dependent upon the haem ligation state and allosteric effectors, such as pH, chloride and bisphosphoglycerate [3].

The spin states and chemical nature of the iron ligation in these proteins may be analysed by EPR spectroscopy. Two families of ferric forms have been reported; the $d_{5/2}^5$ state, with five electrons on the d -orbitals and a total spin of $5/2$, and the $d_{1/2}^5$ state, with two pairs of electrons coupled and a total spin of $1/2$ [4]. The form with a spin of $5/2$ (HS) has a water molecule as the sixth ligand and an EPR spectrum with $g = 6$ and $g = 2$ signals when an X-band spectrometer is used [4,5]. The form with a spin of $1/2$ (LS) has, under the same experimental conditions, two effective g -factors greater and one g -factor smaller than $g = 2$ [6]. The LS forms can only occur in a strong ligand field, when the energy separation between the upper group of d orbitals (z^2

and $x^2 - y^2$) and the lower group (xy , yz and xz) is too big for an electron to exist in the upper orbitals. The EPR characteristics of LS forms depend on the nature of the ligand at the sixth co-ordination position.

MetHb is mainly in an HS form at room temperature. When LS forms occur at room temperature (e.g. in the alkaline metHb species) the UV-visible spectrum shows characteristic 540 and 560 nm bands with a diminution in the HS charge-transfer 635 nm band. More LS forms of metHb are present at low temperature. At least two LS 'acid' forms have been seen at low temperature by EPR spectroscopy, in addition to the usual LS alkaline form [7–9]. The two forms include one with a $g_x = 2.8$ signal postulated to be a form in thermal equilibrium with the normal acid or neutral HS species; the other with $g_x = 2.98$ has been seen during slow cooling only and has been attributed to a true bis-histidine state, perhaps involving a true protein conformational transition. It has been suggested that this $g_x = 2.98$ species is largely due to the β subunits, since β_2 dimers are partly LS at room temperature, while α_2 and $\alpha\beta$ dimers remain HS. In oxyHb the α subunits have hydrogen bonds between the bound O_2 and the distal histidine, but the β subunits do not [10].

In the present study, we have performed a detailed re-examination of the low-temperature EPR spectra of human metHb and compared the results with those obtained using horse metMb. Two pH-dependent LS haem forms are identified in frozen samples of metMb. The proportion of both of these species varies between pH 6 and pH 9. They are assigned to the

Abbreviations used: DTPA, diethylenetriaminepenta-acetic acid; HS, high-spin; LS, low-spin; Mb, myoglobin; PDB, Protein Data Bank.

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classical alkaline metMb and a histidine hemichrome metMb. An additional LS form is seen in purified metHb; this form also occurs in frozen human whole blood.

EXPERIMENTAL

Purified protein samples

Hb and Mb samples (Sigma–Aldrich, Poole, Dorset, U.K.; M1882 horse heart Mb and H7379 human Hb) were dissolved in 100 mM potassium phosphate buffer (pH 6.0) to a haem concentration of 1.5 mM. A further set of human metHb samples was prepared in a similar way using protein purified from a healthy donor's blood according to the method of Antonini and Brunori [2]. Results with the two types of Hb were essentially identical.

The samples were fully oxidized by addition of 2 mM ammonium persulphate and then passed down a Sephadex G-25 column. Final concentrations were measured on a Hewlett Packard 8453 diode-array spectrophotometer using the ferrous CO complexes as standards. Aliquots were reduced by addition of 10 mM dithionite, and then bubbled with CO for 30 s. The molar absorption coefficients for the CO–haem forms, used to calculate the haem concentrations, were: $\epsilon_{540} = 15.4 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for horse Mb–CO and $\epsilon_{540} = 13.4 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for human Hb–CO [2].

Oxidized stock solutions were diluted to approx. 110 μM in 100 mM potassium phosphate buffer at pH 6, 7, 8 or 9. All buffers contained 20 μM diethylenetriaminepenta-acetic acid (DTPA) as a free-iron chelating agent.

Human blood samples

Whole arterial blood was obtained from the radial arteries of several patients suffering from sepsis. Control arterial blood samples were collected from healthy patients undergoing elective orthopaedic surgery. The study protocol was carried out in accordance with the Declaration of Helsinki of the World Medical Association and approved by the local Ethics Committee (Mid-Essex). All patients gave their informed consent.

Aliquots (0.5 ml) were transferred to EPR tubes by means of a long wide-bore blunt-ended needle attached to the disposable syringe used for collection. The samples were frozen within 1 min of collection by gradual immersion of the EPR tube in methanol, cooled in liquid nitrogen to a syrupy consistency (just above freezing point). The samples were then immediately transferred to liquid nitrogen (77 K) and stored at that temperature until the EPR measurements were carried out.

EPR sampling and measurements

Wilma SQ EPR tubes (Wilma Glass, Buena, NJ, U.S.A.) were used for EPR samples. All EPR tubes used had an internal diameter between 3.98 and 4.12 mm. Tubes containing protein solutions, human blood or water (blanks) were quickly frozen in methanol, kept on dry ice. Once frozen, samples were transferred to liquid nitrogen where they were stored prior to measurements. The EPR spectra of either protein solutions or blood were not affected by the time of storage in the liquid nitrogen (measurements after 2 years storage being the same as those after 1 day of storage). The spectra were measured on a Bruker EMX EPR spectrometer equipped with an Oxford Instruments liquid helium system. A spherical high quality Bruker resonator SP9703 was used. The instrumental conditions for the EPR measurements, where not otherwise stated, were: microwave power, $P = 0.8 \text{ mW}$; microwave frequency, $\nu = 9.4865 \text{ GHz}$; modulation

frequency = 100 kHz; modulation amplitude, $A_m = 5 \text{ G}$ (where $1 \text{ G} = 10^{-4} \text{ T}$); sweep rate, $R = 23.8 \text{ G/s}$; time constant, $\tau = 0.082 \text{ s}$; number of scans, $N = 2$ (per spectrum); temperature = 25 K. The concentrations of the different metHb forms in blood were determined using metHb standards under non-saturating conditions (microwave power of 0.05 mW and a temperature of 25 K).

Quantification of EPR signals

We have developed a method to quantify the concentrations in a given sample of paramagnetic species with overlapping EPR signals [11]. Briefly, instead of using the pure line shape signals of each centre, the total EPR spectrum is integrated twice. This double integration is performed on a number of different spectra containing different proportions of the species of interest. A linear system of equations can then be solved to find the integrals of the individual signals. These values of the second integrals are then corrected for the temperature and power saturation behaviour of individual signals. For field-swept EPR spectra, the second integral of the signals should be divided by a factor, which is a function of the effective g -values to make such signals proportional to the concentration [12]. These factors were calculated for all the EPR signals observed and taken into account when the concentrations were calculated. An additional correction factor was applied to the HS haem signal, to account for the fact that at temperatures greater than 10 K the second and third Kramers doublets are also populated [13].

Simulation of relative proportions of HS and LS species in EPR samples

The simulation of the data in Figure 7, using the equations outlined in the discussion was performed using a Microsoft Excel 97 spreadsheet. The equilibrium constants (parameters of the simulation) were found by Microsoft Excel Solver which uses the Generalized Reduced Gradient non-linear optimization code to determine the maximum or minimum value of one cell by changing other cells of the spreadsheet. We used Solver to find the values of the equilibrium constants which corresponded to the minimum of the following sum:

$$L = \sum_{j=1}^N \sum_{i=1}^4 (E_{ij} - S_{ij})^2$$

where E and S are species concentration in the experiment and simulation respectively; i varies from 1 to 4, corresponding to the four pH values, 6, 7, 8 and 9; j varies from 1 to N , where N is the number of species detected in each protein: $N = 3$ for metMb, i.e. HS, LS3 and LS4; and $N = 4$ for metHb, i.e. HS, LS1 (analogue of LS4 in metMb), LS2 and LS3. Therefore the least-square parameter, L , was found for the sum of 16 terms for metHb and for the sum of 12 terms for metMb.

RESULTS

Qualitative analysis

The EPR spectra for metHb and metMb at equal haem concentrations and three different pH values are shown in Figure 1 (in full) and Figure 2 (the LS region). The effective g -factors of the HS forms of metHb and metMb are presented in Table 1. Since the actual $g_{x,y}$ -values of the HS forms lie between those measured at the zero crossing of the derivative and at its peak maximum, $g_{x,y}$ -values calculated by both methods are presented in Table 1. The rhombic distortion in the almost axial (tetragonal) symmetry

Figure 1 EPR spectra of methHb and metMb at 25 K

The haem concentration of all preparations was 110 μ M. The buffer contained 100 mM potassium phosphate and 20 μ M DTPA at various pH values (measured at 25 $^{\circ}$ C).

Figure 2 The LS region of the EPR spectra of methHb and metMb at 25 K

The g -factors of the LS forms indicated are listed in Table 2. Experimental conditions were as described in Figure 1 and the Experimental section. The fluoride (10 mM) complexes of methHb and metMb show no LS forms and a doublet splitting of the $g = 2$ HS component due to ^{19}F .

Figure 3 Comparison of the LS regions of methHb EPR spectra at pH 6 and at pH 7

(a) The superimposed spectra of three independent measurements of a single sample of methHb at pH 6. (b) The superimposed spectra of three independent measurements of a single sample of methHb at pH 7. (c) The superimposed averaged spectra of methHb at pH 6 and pH 7; comparison with (a) and (b) clearly indicates that the differences between the spectra at pH 6 and pH 7 are much greater than any instrumental error or noise. The main LS haem form at pH 7 is LS2, whereas there is a significant contribution from LS1 at pH 6. Conditions and Hb samples were as described in Figure 1 and the Experimental section. Temperature = 25 K.

Table 1 The effective g -factors of the HS forms of methHb and metMb at pH 6–9, measured at 25 K

g -factor	methHb				metMb			
	pH 6	pH 7	pH 8	pH 9	pH 6	pH 7	pH 8	pH 9
g_{xy} (zero line crossing)	5.82	5.82	5.82	5.83	5.84	5.85	5.85	5.87
g_{xy} (peak maximum)	5.96	5.96	5.97	5.96	5.98	5.97	5.97	5.97
g_z	2.000	1.999	2.000	1.999	1.998	1.998	1.998	1.999

Table 2 The effective *g*-factors of the LS forms of metHb and metMb between pH 6 and 9, measured at 25 K

<i>g</i> -factor	metHb			metMb	
	LS1	LS2	LS3	LS3	LS4
<i>g_x</i>	2.7236	2.7853	2.5860	2.5950	2.7000
<i>g_y</i>	2.2410	2.2593	2.1795	2.1615	2.2113
<i>g_z</i>	1.73–1.75	1.65–1.66	1.8319	1.8414	1.756

Table 3 The partial concentrations (%) of the individual forms of haem in the metHb and metMb preparations at pH 6–9 measured by low-temperature EPR spectroscopy

Values represent means $\pm$ S.D. ($n = 6$).

	Partial concentration of haem forms (%)				
	HS	LS1	LS2	LS3	LS4
MetHb					
pH 6	66.1 $\pm$ 2.3	11.1 $\pm$ 1.9	19.6 $\pm$ 3.0	0	0
pH 7	63.9 $\pm$ 1.6	3.4 $\pm$ 0.3	29.6 $\pm$ 1.5	8.1 $\pm$ 1.7	0
pH 8	34.9 $\pm$ 1.2	0	15.2 $\pm$ 0.2	56.9 $\pm$ 1.0	0
pH 9	10.7 $\pm$ 0.8	0	3.9 $\pm$ 0.1	96.8 $\pm$ 1.0	0
MetMb					
pH 6	85.8 $\pm$ 2.7	0	0	0.4 $\pm$ 0.4	13.2 $\pm$ 1.8
pH 7	96.1 $\pm$ 2.4	0	0	0.8 $\pm$ 0.3	3.1 $\pm$ 1.4
pH 8	93.3 $\pm$ 2.1	0	0	3.5 $\pm$ 1.0	2.1 $\pm$ 1.2
pH 9	73.1 $\pm$ 1.4	0	0	15.3 $\pm$ 1.6	0.7 $\pm$ 1.0

of iron in HS metMb is slightly greater than the averaged rhombic distortion for the two metHb subunits; the overall width of the HS signal, from the $g = 6$ region down to the $g = 2$ region, is slightly larger in metMb than in metHb at all four pH values studied. This is consistent with the previously reported differences in $g_{x,y}$ between metHb and metMb [13]. Although the overall linewidth of the HS signal is larger in metMb, the average peak-to-trough width of the signal's $g = 6$ component is sharper in this protein (48 G) than in metHb (52 G), possibly reflecting the presence in metHb of two close but not identical signals, originating from two subunits or two protein conformations.

The LS forms detected in the metHb spectra (cf. Figure 2) are shown in greater detail in Figure 3. When the spectra at pH 6 and pH 7 are superimposed (Figure 3c), it is clear that two distinct species are involved, the relative populations of which are pH-dependent in a pH region where no alkaline form (LS3 type) is to be seen. The effective *g*-factors of these two signals, LS1 and LS2 (Table 2), are close to the values for histidine hemichromes (H-type, where H stands for histidine, according to the nomenclature suggested by Blumberg and Peisach [6]). The strongly pH-dependent LS3 signal has *g*-factors typical of alkaline Hb [6,8] or alkaline Mb [14]; ionization of the distal water molecule produces an LS O-type form (where O stands for oxygen in the hydroxyl anion) [6] in which OH⁻ is co-ordinated to the haem iron. Only two LS forms are seen in the metMb spectra (Figure 2). Of these, one has *g*-values essentially identical to those of LS3 in metHb. The pH dependence of this signal confirms that it represents the alkaline metMb haem form. The other, LS4, resembles the metHb LS1 component.

The LS species observed are unlikely to be due to non-specific protein denaturation during the freezing process; the addition of fluoride, a weak-field ligand, forces the haem to remain HS at all

Figure 4 The EPR spectra of human blood compared with that of purified metHb

The control arterial blood spectrum was obtained by averaging the six spectra from six patients undergoing elective orthopaedic surgery. The spectrum of blood of the patients with sepsis was obtained by averaging spectra from 11 patients. Purified 109 μ M metHb was prepared at pH 8. Microwave power = 3.18 mW; temperature = 10 K. The additional signal at $g = 2.053$ in the blood spectra is caused mainly by the Cu²⁺ ions of ceruloplasmin [37,38]. In the blood spectra, there is a free radical signal overlapping the $g = 2$ component of the HS metHb the latter is clearly seen as a pure component in the metHb preparation. The nature of this free radical was studied in [16].

temperatures. Under these conditions no LS species are observed in the low temperature EPR (Figure 2).

Some other EPR-detectable species are present in these samples. The $g = 4.3$ signal seen in the metHb preparation, and

Table 4 The concentration of different Hb forms in the arterial blood of patients with sepsis and control patients

The difference between the sepsis and control groups is significant for absolute concentration of all Hb forms ($P < 0.006$); it is insignificant for relative concentrations expressed in %.

Hb form	Sepsis		Control	
	Mean $\pm$ S.D. (μ M) ($n = 11$)	Average (%)	Mean $\pm$ S.D. (μ M) ($n = 6$)	Average (%)
HS	16.1 $\pm$ 6.2	72	7.06 $\pm$ 2.8	74
LS1 + LS2	2.2 $\pm$ 0.6	10	0.8 $\pm$ 0.4	8
LS3	4.1 $\pm$ 0.7	18	1.7 $\pm$ 1.4	18
Total	22.4 $\pm$ 6.8	100	9.56 $\pm$ 3.1	100

very faintly in the metMb sample, derives from the non-haem rhombic iron [15]; this represents spontaneous haem degradation or adventitious iron. Its signal intensity was very low, and therefore it was ignored in making quantitative estimates. There is also an almost symmetrical line at $g = 2.065$ in metHb and $g = 2.066$ in metMb. This does not belong to any of the LS states. Although small in size, it is not a cavity artefact. When the background signals are properly subtracted, our spectra (Figures 1 and 2) still contain this signal. Its intensity is pH-dependent in both metHb and metMb, being stronger at pH 5 and decreasing as the pH increases to pH 9 (Figure 2 shows this tendency in the range pH 6–8) which makes this pH dependence similar to that of the LS1 and LS4 forms. However, we are not in a position to speculate on whether this signal is associated in some way with the LS1 and LS4 species.

Quantitative evaluation

The concentration of the HS and LS forms detected in both proteins was found using the deconvolution procedure described in the Experimental section. Table 3 summarizes the concentrations of the several HS and LS forms in the two proteins at four different pH values.

Occurrence of LS Hb forms *in vivo*

The LS haem forms described above are not an artefact of enzyme purification or denaturation as they can be detected in whole human blood. In normal human blood, the LS forms are difficult to detect, given the low overall metHb concentration. However, patients suffering from sepsis are characterized by an elevated concentration of metHb. The result of analysis of arterial blood samples taken from 11 patients with septic shock were compared with those from six control patients (Figure 4). The metHb concentration in the arterial blood from the control patients was similar to that we have previously reported for venous blood from healthy donors [16,17]. The total metHb concentration in the arterial blood from the septic shock patients was significantly raised (Table 4). However, the relative proportion of HS and LS signals was identical to the control patients.

DISCUSSION

Minor non-haem forms

The almost symmetrical line at $g = 2.065$ in metHb and at $g = 2.066$ in metMb does not belong to any of the LS states. We have observed a similar symmetrical singlet in the EPR spectrum of a non-haem peroxidase [18]. Biological EPR signals with g -factors close to 2 are usually interpreted as free radicals. But rheumatoid

bone samples contain a $g = 2.055$ signal identified as a ferric complex [19]. Some inorganic ferric complexes are also characterized by such an EPR parameter. Examples include ferric salts doped with hydrated AlCl_3 [20], ZnO [21] and ZnWO_4 crystals [22]. There are two probable reasons why this signal has apparently not been reported before for metHb or metMb. Firstly, it is very weak, and secondly, it is close to the copper signal common in the background spectrum of almost every resonator. So, for example, an EPR signal with similar properties seen in nitric oxide synthase was attributed to ‘impurities in the cavity and Dewar assembly’ [23]. Our signal is probably due to a ferric complex that is either a haem degradation product distinct from rhombic iron or an adventitious iron atom bound with a low occupancy at a special protein site.

The HS forms

The rhombic distortion in the almost axial symmetry of iron in HS metMb is slightly greater than that averaged for metHb (Table 1). In isolated ferric Hb α chains, the HS form departs from tetragonal symmetry ($g_x = 6.18$ and $g_y = 5.78$) unlike intact metHb, where $g_x \approx g_y$ for all four chains [5]. The physical basis for these differences is not obvious from current structural data. The haem environments are similar for all three monomers (two in metHb and one in metMb); their structures are almost superimposable (Figure 5). The most marked difference is that one propionate side-chain of metMb Haem is held in a different conformation from those of the metHb α and β chain haems. It is hydrogen-bonded to a second [F7 (the seventh residue in the F helix), residue 97] histidine in the proximal pocket, which is replaced by leucine-91 and leucine-96 in α and β Hb subunits respectively (Figure 5). Such replacement apparently prevents formation of the hydrogen bond with the propionate side-chain.

Although the total overall width of the metMb HS signal is greater than that of metHb, its average peak-to-trough width of the $g = 6$ component (46 G) is less than in metHb (52 G). This could indicate the presence of two superimposed signals from α and β subunits in metHb, with closely similar but non-identical parameters. Another possibility is that the R and T states of Hb are characterized by slightly different parameters of the HS EPR signal. However, this seems an unlikely explanation, as the vast majority of the met form would be expected to have the R state structure and thus R/T state differences should not contribute significantly to spectral heterogeneity.

The LS forms and their pH dependencies

The LS haem g -factors are defined by two symmetry characteristics of their environment: rhombicity and tetragonality [6]. Whereas the overall width of a HS signal (Figure 1), in particular

A **B**

Figure 5 The haem environments and haem groups of metHb and metMb

(A) Haem environments. The haem groups are shown edge on with the distal region above and the proximal region below the haems. The right-hand structures are rotated 80° with respect to the left-hand structures. (B) Haem groups. The apparent configurations for the five haem groups alone. The right-hand structures are rotated 90° with respect to the left-hand structures. Hb co-ordinates for human T-state aquometHb; Protein Data Bank (PDB) file 1HGB [28]. Mb co-ordinates for wild-type horse heart recombinant Mb; PDB file 1WLA [27]. The structures were superimposed and the haem group environments were fitted using Swisspdbviewer 3.0 ('Magic Fit'). Non-identity of the two α subunits and the two β subunits of metHb may be a consequence of the precision of structure determination involved.

Figure 6 Changes of effective g -factors as a function of unpaired electron density

The g_x , g_y and g_z values for the different LS forms of metHb and metMb are plotted along an axis representing unpaired electron density. The EPR signals LS1, LS2, LS3 and LS4 are shown in Figure 2 and their g -factors used to create this Figure are tabulated in Table 2.

the splitting of the g_x and g_y components, is a measure of rhombicity, the width of a LS signal is usually a measure of tetragonality. Rhombicity is a function of the geometrical arrangement of the ligands and the π -bonds between them and the iron atom. Tetragonality depends upon the charge on the

iron atom. The latter, in turn, depends on the sum of the electronegativities of the z -axis ligands [6]. The greater the electron donation to the iron, the smaller the unpaired electron density upon it, and hence the less the tetragonality. Conversely, the more unpaired electron density upon the haem, the broader and less isotropic the signal. As the density on the ligand (or the time the unpaired electron spends on it) increases, the effective g -factors come closer. In the limit, when 100% unpaired electron density is on the ligand and electron donation to Fe(III) is also 100%, this effectively reduces it to a diamagnetic Fe(II) state, and the three g -factors equal the g -factor of a free radical, i.e. 2.00 (Figure 6).

With the fifth (proximal histidine) ligand fixed, the tetragonality of metHb and metMb LS forms depends upon the electronegativity of the sixth ligand. The nature of this ligand can have a profound effect on the g -factors of the LS forms; the widest, the 'C' type, has a sulphur atom of methionine as the sixth ligand and $g_x \approx 3.15$ [6], and the narrowest is an unstable product of the one-electron reduction of oxyHb α subunits where $g_x \approx 2.20$ [24]. The major LS form at pH 8, which we have called LS3, is the classical alkaline species. This has hydroxide ligation. LS1, LS2 and LS4 all have g -values typical of bis-histidine ligation and we assign these to histidine hemichromes. As hydroxide is more electronegative than histidine, more unpaired electron density is transferred from the iron to the hydroxide ligand. This has the effect of sharpening the spectrum and results in a narrower range of g -values (Figure 6).

As the ligands are the same, the differences in g -factors we observe in the different bis-histidine LS forms of metHb and metMb must reflect relatively subtle variations in the haem geometry. The simplest explanation is a change in the distance, D ,

Scheme 1 Scheme showing proposed pH-dependent and pH-independent changes at the haem groups

Protonation/deprotonation of a hypothetical site X on the globin (metHb or metMb) results in a conformational switch in the protein: $A \leftrightarrow B$ (pK_{AB}) when iron is in the HS state and $A' \leftrightarrow B'$ ($pK_{A'B'}$) when a water molecule is excluded from the haem pocket and therefore iron is LS. The water molecule exclusion/addition from/to the pocket is governed by pH-independent equilibrium constants $K_{AA'}$ and $K_{BB'}$ defined separately for the two conformations when X is protonated and when it is not. The water molecule at the sixth position is deprotonated at pK_{BC} , resulting in the alkaline met haem form (LS3) in both proteins. In the HS haem state the iron atom is effectively larger than in the LS forms, and hence it is slightly (0.5 Å) displaced from the porphyrin plane towards the fifth ligand, the proximal histidine [39].

Figure 7 Simulation of the pH dependencies of HS and LS forms of methHb (A) and metMb (B)

The symbols represent the means $\pm$ S.D. for six samples made for each pH value (numerical values are given in Table 3). The lines are the simulated solutions (3) of the system of equations (1). System (1) describes the mechanism proposed in Scheme 1. The values of the equilibrium constants used to attain the best-fit to the experiment were found as described in the Experimental section and are presented in Table 5 (experiment 3). The HS form concentration measured in the experiment corresponds to the sum of the concentration of states A and B. Similarly LS1 and LS2 in methHb correspond to states A' and B' respectively, LS4 in metMb corresponds to state A', and LS3 in both proteins corresponds to state C. The simulated line for LS2 in metMb (B' state in metMb) is indistinguishable from the x-axis which corresponds to the fact that this state is not significantly populated in metMb.

between the distal histidine and the haem iron, such that $D_{LS2} > D_{LS1} > D_{LS4}$. The least anisotropic (narrowest) spectrum among the histidine hemichromes is LS4; therefore LS4 has the greatest unpaired electron density on the histidine. This is consistent with the nitrogen atom being closest to the haem in this form. The LS2 signal has the most anisotropic (widest) spectrum, and the metHb species responsible will consequently have the least unpaired electron density on the ligand. Thus LS2 represents the most distant location of nitrogen from the haem iron for the histidine hemichromes. The distance between nitrogen and haem iron in LS1 is an intermediate one, being closer to the distance in LS4 rather than that in LS2 (Figure 6).

The proportions of the HS and all LS species observed (not just the alkaline LS3 form) are strongly pH-dependent in the pH range 6–9. Clearly there must be some other protonatable group that can affect the HS–LS transition and the nature of the LS species observed. Although in principle different groups could be protonated in the HS and different LS species, the simplest model would be one that could explain all the observed spectra with just one protonatable group on the globin.

In this model when metHb or metMb is in the HS haem state, a site, X, on globin is protonated/deprotonated with an equilibrium constant K_{AB} (Scheme 1). Protonation of X results in a change of the protein hydrogen bond network; this in turn causes a conformational change, such that the distal histidine moves closer to the iron atom (compare states A and B in Scheme 1). Both states, A and B, can lose the water molecule in the sixth co-ordination position (pH-independent equilibrium constants $K_{AA'}$ and $K_{BB'}$) forming states A' and B' respectively. The latter are the LS states, both distal histidine hemichromes, but with slightly different distances between imidazole and iron. The A' and B' states are themselves in a pH-dependent equilibrium ($K_{A'B'}$).

As discussed above, in LS1, the distal histidine is closer to the haem than in LS2, and thus LS1 is state A' and LS2 is state B'. The only histidine hemichrome detected in metMb, LS4, has an EPR spectrum with the effective g-factors closer to those of LS1 rather than those of LS2 in metHb. We therefore assign LS4 to state A' as well.

The distal water ligand in HS metHb and metMb can also deprotonate (state C in Scheme 1), generating the alkaline LS form. The pK values for this transition measured optically for these proteins are approx. 1 unit different; pK = 8.05 for human metHb [25] and pK = 8.93 for horse metMb [26]. Such a big difference in the pK values in two proteins may reflect different distances from the water molecule to the haem iron. The closer to the haem, the more readily the water molecule can lose a proton. The distance from the distal histidine to the haem in metMb (4.41 Å [27]) is greater than that in both α and β subunits of metHb (4.28 and 3.98 Å respectively [28]); thus if the water molecule were to occupy a position midway between histidine and haem, it would be expected to be closer to the metHb haems and deprotonate more readily.

The equilibrium of all components shown in Scheme 1 is described by the following system:

$$\left. \begin{aligned} K_{AB} &= \frac{BH}{A} \\ K_{BB'} &= \frac{B'}{B} \\ K_{A'B'} &= \frac{B'H}{A'} \\ K_{AA'} &= \frac{A'}{A} \\ K_{BC} &= \frac{CH}{B} \end{aligned} \right\} \quad (1)$$

$$A + B + C + A' + B' = T$$

where A, B, C, A' and B' are the concentrations of different haem states (Scheme 1), H is the concentration of protons and T is the total concentration of haem.

Since the first four constants are the equilibrium constants in a cycle, an additional condition applies:

$$K_{AB}K_{BB'} = K_{A'B'}K_{AA'} \quad (2)$$

Table 5 The values of the equilibrium constants described in Scheme 1 and obtained as the best-fit simulation of the experimental pH dependences of the HS and LS haem forms detected in metHb and metMb

The results of three individual simulations corresponding to three independent experiments are presented together with the mean values ($\pm$ S.D.).

Constant	Defined as:	metHb				metMb			
		Experiment number				Experiment number			
		1	2	3	Mean $\pm$ S.D.	1	2	3	Mean $\pm$ S.D.
pK_{AB}	$-\log(BH/A)$	5.58	5.84	5.66	5.69 ± 0.13	6.34	6.33	5.70	6.12 ± 0.36
$K_{BB'}$	B'/B	0.38	0.43	0.49	0.43 ± 0.05	0.01	0.01	0.04	0.02 ± 0.02
$pK_{A'B'}$	$-\log(B'H/A')$	5.87	5.76	5.62	5.75 ± 0.13	7.60	7.83	6.76	7.40 ± 0.56
$K_{AA'}$	A'/A	0.75	0.36	0.44	0.52 ± 0.21	0.22	0.16	0.41	0.26 ± 0.13
pK_{BC}	$-\log(CH/B)$	7.87	8.00	7.79	7.89 ± 0.10	9.21	9.49	9.59	9.43 ± 0.20

The solutions of system (1) were found to be as follows:

$$\left. \begin{aligned} \frac{A}{T} &= \frac{H}{K_{AB}Z} \\ \frac{B}{T} &= \frac{1}{Z} \\ \frac{A'}{T} &= \frac{K_{BB'}H}{K_{A'B'}Z} \\ \frac{B'}{T} &= \frac{K_{BB'}}{Z} \\ \frac{C}{T} &= \frac{K_{BC}}{HZ} \end{aligned} \right\} \quad (3)$$

$$\text{where } Z = 1 + K_{BB'} + \frac{H}{K_{AB}} + \frac{K_{BC}}{H} + \frac{K_{BB'}H}{K_{A'B'}}$$

The solutions above are the concentrations of different haem forms shown in Scheme 1 and are expressed in units relative to the total protein concentration. These solutions depend exclusively on proton concentration (pH) and on equilibrium constants. We can therefore use the experimentally determined proportions of these species at different pH values to determine the equilibrium constants (see the Experimental section). The sum of states A and B were considered to be the observed HS haem concentration; this assumes that the line shape of the HS haem EPR signal is insensitive to the protonation state of site X. This assumption is more valid for metHb, than for metMb, since the line shape of the HS signal was identical in metHb at all four values of pH, while we detected a subtle change in the metMb HS signal shape at different pH values (although this was still small enough to ignore in quantitative estimates).

The good fit of the simple model and experimental data (Figure 7) means that we do not need to consider a more complicated model. The equilibrium constants used to achieve the best fit to the experiment are given in Table 5. The pK value for the proton at site X when haem is in the HS state, pK_{AB} , is similar for both metHb and metMb, 5.69 ± 0.13 and 6.12 ± 0.36 respectively, indicating that species X is likely to be the same in the two proteins. This value does not change significantly for metHb when the water molecule is lost from the haem co-ordination leading to LS histidine hemichrome formation ($pK_{A'B'} = 5.75 \pm 0.13$). However, when metMb loses water, the pK value for the proton at site X becomes significantly more alkaline ($pK_{A'B'} = 7.40 \pm 0.56$). Thus the LS histidine hemichromes in metMb (LS4 and LS2) are characterized by a significantly different globin conformation than similar hemichromes in

metHb (LS1 and LS2), making it easier to protonate site X in metMb.

For both proteins $K_{AA'}$ is greater than $K_{BB'}$. Thus the water molecule is displaced more readily from state A than state B. This is consistent with the proposed proximity of the distal histidine to the haem in state A, compared with state B (Scheme 1). This effect, however, is much more pronounced in the case of metMb; the value of $K_{BB'}$ in metMb is approx. 20-fold lower than in metHb. This might be a result of differences in water molecule co-ordination in the haem pocket in metMb and metHb; it has been suggested that other amino acids, not simply distal histidine, can ligate the water in haem proteins [29].

The pK_{BC} values obtained from the simulation for metHb and metMb correspond well to the pK values reported for the 'acid-alkaline' transtion for these two proteins [2]. The effective pH of our buffer solutions at 25 K is likely to be approximately 0.5 pH units more acid than that measured at room temperature [30,31]. The pK values in Table 5 may therefore require adjustment. The minimal estimated decrease in pH by 0.5 units at 25 K compared with 300 K would give 'true' pK_{BC} values of 8.93 for metMb and of 7.39 for metHb. A $pK_{BC} \approx 8.93$ would be typical for metMb; that of ≈ 7.39 for metHb would be lower than at 300 K and suggest that, in contrast to Keilin and Hartree [32], the transition of metHb to the alkaline form is actually promoted at low temperature.

MetHb has previously been reported to show two LS 'acid' forms at low temperatures, in addition to the alkaline form [7–9]. The form with a signal at $g_x = 2.8$ was proposed to be in thermal equilibrium with the normal acid or neutral HS species. This is almost certainly the same as the LS2 form ($g_x = 2.785$) described in the present study. Levy et al. [8] and Tsuruga et al. [9] also describe another LS form with $g_x = 2.98$, attributed to another bis-histidine state formed during slow, but not fast, cooling. We have not seen such a form under our experimental conditions. The cooling rate may be a significant parameter determining the ultimate concentration of this form; the LS2 form may be the only extra state in thermodynamic equilibrium with the major HS species.

We have interpreted the acid pK_{AB} , having close values in the two haemoproteins, as the protonation of a site, X, distant from the haem iron. This induces a conformational change that changes the distance between the iron and the distal histidine. Could it be that it is the distal histidine itself that is protonated? It has been reported that the distal histidine protonation is coupled to a conformational equilibrium between 'open' and 'closed' distal pocket states, which have different rates of diatomic ligand exchange that differ by an order of magnitude

[33]. pH-dependent hydrofluoric acid binding to metMb was also interpreted in terms of distal histidine protonation at acidic pH [34]. This possibly general effect of facilitating diatomic ligand binding to the haem when the distal histidine is protonated, might be associated with the histidine swinging out of the pocket at pH 4 and thus opening a direct channel from the solvent to the iron [35,36]. However, we think it is unlikely that the ionisation at pK_{AB} (and $pK_{A'B'}$), reported in the present study is associated with the distal histidine. First, our values (Table 5), even when corrected for the low temperature [30,31], are still higher than the pK values reported for the distal histidine ($pK \approx 4$) [33,34]. Second, a protonated imidazolium ring cannot co-ordinate haem iron. Therefore if the ligand in LS1 and LS4 is indeed the distal histidine, as suggested by the EPR data, then the group with pK_{AB} (Scheme 1 and Table 5) must lie elsewhere in the molecule.

Relevance to whole human blood

MetHb represents approx. 0.3% of the total blood Hb [1]. A significant fraction of this is in the LS form when measured using low temperature EPR (Table 4). This demonstrates that the LS signals seen in the EPR spectra of the isolated protein are not due to modification of the Hb samples during purification or manipulation prior to freezing; rather they are normal conformational states of the native protein, detectable whenever it is frozen to a low temperature.

The total amount of metHb in the blood of patients with sepsis is more than double that of control patients. However, the relative proportion of the HS and LS forms is constant (Table 4). The HS/LS ratio observed in blood is similar to that expected for a sample of purified metHb, frozen at blood pH (Figure 7A). This indicates that the pH in the sample is the dominant factor determining the HS/LS ratio, both in the pure protein and in whole blood.

The ability to detect such minor Hb forms in intact blood allows us to determine if the thermodynamics of the different states postulated in Scheme 1 vary in different pathologies. In the group of sepsis patients examined in the present study, the level of total metHb in blood increased compared with that found in healthy controls, but the relative populations of different forms of metHb showed no abnormalities. Whether this is true for other pathologies remains to be seen.

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