Cross-linking with O-raffinose lowers oxygen affinity and stabilizes haemoglobin in a non-cooperative T-state conformation

Yiping JIA*1, Somasundaram RAMASAMY+1, Francine WOOD*, Abdü I. ALAYASH2 and Joseph M. RIFFKIND†1

*Laboratory of Biochemistry and Vascular Biology, Division of Hematology, Center for Biologics Evaluation and Research (CBER), Food and Drug Administration (FDA), Bethesda, Maryland 20892, U.S.A., and †Molecular Dynamics Section, National Institute on Aging, National Institutes of Health (NIA), 5600 Nathan Shock Drive, Baltimore, Maryland 21224-6823, U.S.A.

O-R-polyHbA0 is an intra- and intermolecularly O-raffinose cross-linked derivative of deoxygenated human haemoglobin developed as an oxygen therapeutic. When compared with its native protein (HbA0), O-R-polyHbA0 was found to be locked in the T (tense) quaternary conformation with a lower oxygen affinity, a reduced Bohr effect (50 % of HbA0) and no measurable cooperativity (h = 1). The kinetics of oxygen and CO binding to the protein indicate lower ‘on’ rates and faster ‘off’ rates than HbA0, and the absence of effects of inositol hexaphosphate (IHP) on the kinetics. Other properties consistent with a T-like conformation are inaccessibility of the βCys-93 thiol group of O-R-polyHbA0, the hypofluorescent splitting from nitrogen in the EPR spectrum of the Fe(II)NO complex of O-R-polyHbA0, and decreased flexibility in the distal haem pocket, as indicated by low-spin bis-histidine complexes detected by EPR of oxidized chains. A comparison of the properties of O-R-polyHbA0 with those of HbA0 with and without IHP, as well as the reaction of nitrite with deoxygenated haemoglobin, provide additional insights into the variations in the conformation of T-state haemoglobin in solution (modifications of the T state produced by adding organic phosphates, like IHP and 2,3-diphosphoglycerate). Although the physiological ramifications of locking HbA0 in the T conformation with the O-raffinose are still unknown, valuable insights into haemoglobin function are provided by these studies of O-R-polyHbA0.

Key words: blood substitute, haemoglobin, modified Hb, oxygen transport, T state.

INTRODUCTION

Haemoglobin exists in two quaternary states, a low affinity T (tense) state and a high affinity R (relaxed) state. Binding of oxygen by haemoglobin in the T state causes tertiary conformational changes that shift the equilibrium towards the R state, cooperatively increasing the affinity of other subunits [1,2]. The last few decades, investigations of the structure–function relationships in haemoglobins have been greatly enhanced by the use of naturally occurring mutants, site-directed mutants and chemically modified haemoglobins involving key amino acid residues. In some instances, cell-free haemoglobins chemically altered or genetically expressed in microbial host systems, developed as oxygen carrying therapeutics, have also been used for such investigations [3].

It is, nevertheless, very difficult to differentiate between the tertiary and quaternary conformational changes during haemoglobin oxygenation. For this purpose, methods have been developed to fix the haemoglobin molecule in either the T or R quaternary states. One approach is to stabilize the T quaternary structure by preventing the T→R transition, either by the crystallization of deoxyhaemoglobin from polyethylene glycol solutions or by encapsulation of deoxyhaemoglobin in wet porous silica gels. These external forces prevent the structural transition in the haemoglobin molecule [4]. The advantage of encapsulation of the deoxy form in wet porous silica gels over crystallization is the relatively accurate determination of oxygen binding curves in the presence and absence of allosteric effectors, which cannot be accomplished with crystals [5].

When haemoglobin is cross-linked with a reagent in the ligated form (R state), it inhibits the normal transition to the low affinity form (T state), resulting, in some cases, in a protein with high affinity and reduced cooperativity. In order to enhance off-loading, cross-linkers that stabilize haemoglobin in the T state have, however, been sought for their potential usefulness as oxygen therapeutics [6]. Bis(3,5-dibromosalicyl)umarate, which cross-links the two α subunits at Lys-99 in the deoxy form, stabilizes the T state and has been extensively evaluated as a potential blood substitute both in vitro and in vivo [7,8]. O-Raffinose cross-linked haemoglobin, prepared by the reaction of deoxyHbA0 (where HbA0 is chromatographically purified human haemoglobin) with O-raffinose (a polyaldehyde obtained through oxidation of the trisaccharide raffinose) to give O-R-polyHbA0, is another haemoglobin-based blood substitute currently under clinical evaluation. During the cross-linking process, the aldehyde of O-raffinose combines with the amino groups of HbA0 to form reversible Schiff base linkages. These are then reduced to stable amines. Oxidized raffinose has been speculated to not only stabilize the tetrameric structure of the deoxyHbA0 (T state) by covalently cross-linking the two αβ dimers at the amino groups within the 2,3-diphosphoglycerate (2,3-DPG) binding site, but also to oligomerize the tetramer. The O-R-polyHbA0 solution treated with SDS to dissociate non-covalent bonds that results from this procedure has been reported to consist of the following HbA0 oligomers: 32 kDa dimer (⩽ 5 %); 64 kDa tetramer (33 ± 10 %); 128–600 kDa 2–10 tetramers (63 ± 12 %); ≥ 600 kDa larger oligomers (⩽ 3 %) [9]. New (unpublished) data about our ongoing work to fully characterize this haemoglobin have been mentioned in the Discussion.

Abbreviations used: 2,3-DPG, 2,3-diphosphoglycerate; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); ΔH, enthalpy change; HbA0, chromatographically purified human haemoglobin; IHP, inositol hexaphosphate; metHb, ferric form of Hb; OEC, oxygen equilibrium curve; p50, Po2 at which haemoglobin is half saturated; R (state), high affinity relaxed quaternary (state); O-R-polyHbA0, O-raffinose cross-linked derivative of deoxygenated human haemoglobin; T (state), low affinity tense quaternary (state).

1 These authors contributed equally to this work.

2 To whom correspondence should be addressed (email alayash@cber.fda.gov).
In this study we report the ligand-binding properties of O-R-polyHbA0, which represents one of a few haemoglobin-based blood substitutes in clinical use manufactured from its ultrapure precursor, HbA0. We show that this haemoglobin maintains the T state even after being oxygenated, and is therefore an interesting model system in which the properties of T-state haemoglobin can be investigated without the complications associated with trapping methods to stabilize the T state.

**EXPERIMENTAL PROCEDURES**

**Haemoglobin solutions**

Ultrapure HbA0 and its O-raffinose cross-linked derivative O-R-polyHbA0 (Hemolinink™) were a kind gift of Hemosol Inc. (Mississauga, Ontario, Canada). HbA0 was prepared from outdated human blood and subjected to extensive anionic and cationic chromatographic procedures resulting in a protein with purity greater than 99%. O-R-polyHbA0 was prepared by reacting HbA0 with O-raffinose, a hexaldehyde obtained by oxidation of the trisaccharide raffinose. The high level of purity of the haemoglobin solutions was confirmed by the use of SDS/PAGE analysis, isoelectric focusing, HPLC and Western blotting [9,10]. The ferric (met) forms of haemoglobins were prepared by addition of a 1.2-fold excess of potassium ferricyanide to oxyhaemoglobin. Unreacted ferricyanide and its reaction products were removed by a two-step gel filtration procedure on Sephadex G-25. The first filtration step (high pH, high salt) was done in 50 mM phosphate buffer (pH 8.3), 1 M NaCl. The following step (neutral pH, low salt) was done in 50 mM phosphate buffer (pH 7.0) with no added NaCl.

**Oxygen equilibrium studies**

Oxygen equilibrium curves (OECs) of haemoglobin solutions were obtained using the Hemox Analyzer (TCS Scientific, New Hope, PA, U.S.A.). This instrument measures the oxygen tension with a Clark oxygen electrode (Model 5331 Oxygen Probe; Yellow Springs Instrument, Yellow Springs, OH, U.S.A.) and simultaneously calculates the haemoglobin saturation using a dual-wavelength spectrophotometer. The concentration of haemoglobin was 60 μM (haem) and the temperature was maintained, unless otherwise indicated, at 37 °C. To maintain the metHb content to a minimum (<5%), 4 μl of the Hayashi enzymatic reduction system was included [11] in the final 4 ml Hemox buffer solution (135 mM NaCl, 5 mM KCl, and 30 mM Tes, pH 7.4). Oxygen equilibrium parameters were derived by fitting the Adair equations to each OEC by a nonlinear least-squares procedure included in the Hemox Analyzer software (p50 PLUS, version 1.2). The Adair constants were then used to generate a complete O2-binding curve, which was used to determine the p50 (p50 is the pO2 at which haemoglobin is half saturated) and h (Hill coefficient) for oxygen binding. This procedure made it possible to determine O2-binding curves for samples of the modified haemoglobin, which were not fully oxygenated at atmospheric partial pressures [12].

The dependence of the oxygen affinities of these haemoglobins on pH and temperature was determined according to established procedures [13]. The variation in pH was carried out in mixtures of 0.1 M bis-Tris buffer and in 0.1 M Tris buffer to cover a wide pH range (pH 5.5 to pH 9.0) with constant chloride concentration (0.1 M NaCl) and a constant temperature of 37 °C according to Bucci and Fronticelli [14]. The effects of hypothermic conditions on the oxygen carrying capacity of haemoglobins were investigated in the Hemox Analyzer in conjunction with a refrigerated circulating water bath. The enthalpy change (ΔH) associated with the oxygen binding reaction with haemoglobin was determined by measurements over the temperature range (15–37 °C) in 10 mM potassium phosphate buffer/0.1 M NaCl buffer, pH 7.4. ΔH was calculated from the slopes of the regression lines according to van’t Hoff’s equation [15].

**Rapid kinetic studies**

Oxygen dissociation rates were determined in an Applied Photophysics SF-17 microvolume stopped-flow spectrophotometer, as described previously [16]. Haemoglobin solutions (30 μM) were deoxygenated by rapidly mixing each haemoglobin solution with 1.5 mg/ml sodium dithionite (BDH) and deoxygenation was followed at 437.5 nm in 0.1 M bis-Tris buffer, pH 7.4 at 20 °C. The kinetics of carbon monoxide (CO) combination with deoxyhaemoglobin was carried out in the stopped-flow apparatus as described previously [17]. Experimental conditions were 50 mM bis-Tris buffer at pH 7.4, 100 mM NaCl, at 25 °C. Both haemoglobin solutions and CO solutions containing sodium dithionite were mixed and reactions were monitored at 437.5 nm. The kinetics of oxygen dissociation in fully liganded proteins and the CO binding to the proteins were also carried out in the presence of various concentrations of inositol hexaphosphate (IHP).

**EPR measurements**

EPR spectra were obtained using an IBM ER 200D-SCC spectrometer with 100 kHz modulations at 2 mW power. The temperature was maintained at 4.2 K by an Oxford Instruments ESR-900 continuous flow cryostat. Haemoglobin concentrations for EPR were 1 mM. The samples of nitrosylhaemoglobin (HbNO) were prepared by passing nitric oxide (first slowly bubbled through 1 N NaOH) over a solution of completely deoxygenated haemoglobin for 5 min. The sample was then anaerobically transferred to a 4 mm O.D. quartz EPR tube.

**Reactive thiol content**

Thiol content was determined by incubating protein samples (5 μM to 15 μM in haem) in 50 mM sodium phosphate, pH 7.4, with DTNB [5,5-dithiobis-(2-nitrobenzoic acid)]. Absorbance was then measured at 446 nm to avoid the interference of DTNB absorbance with that of haem at 408 nm [18,19], and the concentration of free thiol was estimated by comparison with GSH standards.

**Reduction of nitrite by deoxyhaemoglobins**

Nitrite (20 μM) was reacted with 100 μM deoxyhaemoglobin in 46 mM NaCl, 4 mM sodium phosphate buffer, pH 7.4, 100 μM EDTA. The formation of haem NO was determined by a Sievers-280 Nitric Oxide Analyzer [20].

**RESULTS**

**Oxygen equilibrium studies**

The OECs of HbA0 and its polymerized version (O-R-polyHbA0) are shown in Figure 1. Polymerization of HbA0 clearly induced changes not only in the position but also in the shape of the OEC with the curve for O-R-polyHbA0 right shifted and less sigmoidal in shape. Shown in Figure 1 is the actual data obtained with the Hemox Analyzer. From the curve for O-R-polyHbA0 it is evident that the sample is not fully oxygenated at the maximum pO2 obtained with this instrument. However, the observed data has been
analysed and a linear Hill plot with \( h = 1 \) was obtained. The absence of any upward curvature, even at 70% oxygenation where three \( O_2 \) molecules are already bound (results not shown), suggests that there is no major conformational change when the haemoglobin is fully oxygenated. We have, therefore, used the Adair equation to fit the observed data and used the Adair constants to calculate \( p^{50} \) and \( h \) (see Experimental Procedures). The calculated value for \( p^{50} \) was 50.9 mmHg compared with that of its native precursor HbA0 (\( p^{50} = 13.5 \) mmHg) under the same experimental conditions (Table 1). The Hill coefficient at half saturation calculated for HbA0 is within the normal range (\( h = 2.6 \)) whereas the value for O-R-polyHbA0 is dramatically reduced (\( h = 1 \)) (Table 1), indicative of a loss of cooperativity.

Figure 2 shows the Bohr effect curves for binding oxygen to HbA0 and O-R-polyHbA0 over a wide range of pHs. A much-reduced Bohr effect is found for the polymerized haemoglobin than for HbA0. The reduced Bohr effect can be quantitated by measuring the Bohr coefficient derived from the slope of these plots between pH 6.5 and 8.0, which is a measure of the proton-linked oxygen release associated with the alkaline Bohr effect. For HbA0, this coefficient was 1.68 protons (H) per tetramer whereas for O-R-polyHbA0 the value decreased to 0.86 H per tetramer. The 50% reduction in the proton-linked oxygen release may reflect the modification of Bohr amino acid residues subsequent to the modification of HbA0 by O-raffinose.

Changes in the \( p^{50} \) with temperature for HbA0 and O-R-polyHbA0 were carried out in the range 15–37 \( ^\circ \)C in the Hemox Analyzer (results not shown). When the log \( p^{50} \) was plotted against the reciprocal value of temperature, a linear relationship was found for both haemoglobins. Application of the van’t Hoff rule gave the following values for the heat of oxygenation: HbA0 (\( \Delta H = -15.8 \) kcal/mol) versus O-R-polyHbA0 (\( \Delta H = -12.5 \) kcal/mol) (Table 1). These values were not corrected for the heat of solution of oxygen (3 kcal/mol). For HbA0, this value is very close to that reported in the literature under similar experimental conditions. The reduced enthalpy value calculated for O-R-polyHbA0 implies that less heat was liberated during the oxygenation and is consistent with the notion that O-R-polyHbA0, unlike HbA0, remains in the T state when oxygen is added and there is no contribution of the quaternary change to the enthalpy of oxygenation.

### Rapid kinetic studies

Rapid mixing kinetic experiments (oxygen dissociation and CO binding) were carried out using a stopped-flow spectrophotometric method to further explore the allosteric properties of O-R-polyHbA0. A single exponential process whose rate is

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**Table 1** Ligand-binding parameters of HbA0 and O-R-polyHbA0

<table>
<thead>
<tr>
<th></th>
<th>( p^{50} ) (mmHg)</th>
<th>( h )</th>
<th>( k_{st} ) (s(^{-1}))</th>
<th>( k_{st} ) (s(^{-1})) + IHP (300 ( \mu )M)</th>
<th>( \Delta H ) (kcal/mol)</th>
<th>( k_{on} ) (l( M )(^{-1} \cdot s ))</th>
<th>( k_{off} ) (s(^{-1}))</th>
<th>IHP (100 ( \mu )M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA0</td>
<td>13.5</td>
<td>2.6</td>
<td>31 ± 0.058</td>
<td>56 ± 0.23</td>
<td>15.8</td>
<td>1.7 ± 0.08</td>
<td>0.7 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>O-R-polyHbA0</td>
<td>50.9</td>
<td>1.0</td>
<td>130 ± 3.5</td>
<td>135 ± 0.9</td>
<td>-12.5</td>
<td>1.2 ± 0.04</td>
<td>1.1 ± 0.06</td>
<td></td>
</tr>
</tbody>
</table>

* The \( p^{50} \) and \( h \) are derived from Figure 1 (see the legend for details).

† Experimental conditions are described in the Figure 3 legend.
31.0 ± 0.06 s⁻¹ can describe the time course observed in the stopped-flow for HbA₀. This value is close to that reported previously for human haemoglobin [13]. For O-R-polyHbA₀, two phases are seen with corresponding amplitudes $A_1 = 0.21 ± 0.0017$ and $A_2 = 0.03 ± 0.002$ (results not shown). Approx. 87% of the reaction at pH 7.4 has a rate of $130 ± 3.5$ s⁻¹, roughly 4 times faster than that obtained for HbA₀ (Table 1). The increase in the oxygen ‘off’ rates in the modified form of HbA₀ parallels the decrease in oxygen affinity ($p_{50}$). The kinetic heterogeneity seen for the dissociation kinetics of O-R-polyHbA₀ may reflect the presence of fast reacting and slow reacting species, a phenomenon known to occur with other polymerized haemoglobins [21]. However, the low amplitude (0.03) for the slow phase implies that the heterogeneity has a relatively small effect on the ligand reactions.

A monophasic time course was obtained for both HbA₀ and its modified form, O-R-polyHbA₀ when the deoxyhaemoglobins were mixed with CO. Second-order rate constants for the binding of CO ($k_{on}$) were calculated from the CO concentration dependence of the rates of CO binding observed in rapid mixing experiments under pseudo first order conditions and are reported in Table 1. As can be seen in this table, in the absence of added IHP, CO binding to the modified protein is slightly reduced when compared with that of the unmodified protein.

Figure 3 shows representative plots of the rate constants for the reactions of HbA₀ and O-R-polyHbA₀ with ligands as a function of IHP as measured in the stopped-flow under standard conditions. Figures 3(A) and 3(B) show oxygen dissociation rates ($k_{off}$) from the oxy forms of HbA₀ and O-R-polyHbA₀ as a function of IHP concentrations, and Figures 3(C) and 3(D) show CO binding kinetics to the deoxy forms of these two proteins with and without IHP. As expected, IHP increased the apparent rate of oxygen dissociation ($k_{off}$) from the oxyHbA₀ (Figure 3(A) and Table 1), but it had little or no effect on the oxygen dissociation from O-R-polyHbA₀ (Figure 3B). When the deoxy form of HbA₀ is rapidly mixed with increasing concentrations of CO in the presence of a saturating amount of IHP (100 µM) there was a several-fold reduction in the rate of CO binding ($k_{on}$), but IHP had no effect on the CO reaction kinetics with O-R-polyHbA₀ (Figures 3C and 3D).

**Thiol content**

βCys-93 is situated in a conformationally plastic domain containing residues whose interactions are directly linked to allosteric properties of the haemoglobin tetramer [2]. The thiol content of both haemoglobins (oxy form) was determined and compared with that of control proteins, horse heart myoglobin (which lacks cysteine), and a recombinant myoglobin in which alanine at position 94 has been replaced by cysteine. This comparison was carried out in order to determine the reactivity of the βCys-93 residue, a well-established index of haemoglobin conformational state in HbA₀, after the O-raffinose modification. It is apparent from Table 2 that horse heart myoglobin lacks thiol reactivity as expected. In contrast, thiol reactivity was clearly present in the recombinant myoglobin (Ala → Cys-94) and HbA₀. There were 6 µM reactive thiol groups in 15 µM HbA₀ solution (haem concentration), i.e. about 1.6 reactive thiol groups per haemoglobin tetramer. In the native haemoglobin molecule, only two thiol groups (β93) are reactive although all six have been reported to be reactive in the denatured haemoglobin molecule [3]. However, O-R-polyHbA₀ lacks the thiol activity under our experimental conditions.
Cross-linking of haemoglobin with O-raffinose

Table 2  Thiol reactivity of haemoglobins and myoglobins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Reactive thiol content (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse heart myoglobin</td>
<td>0</td>
</tr>
<tr>
<td>Sperm whale mutant myoglobin</td>
<td>7</td>
</tr>
<tr>
<td>HbA0</td>
<td>6</td>
</tr>
<tr>
<td>O-R-PolyHbA0</td>
<td>0</td>
</tr>
</tbody>
</table>

Reactive thiol content was determined by measuring the absorbance at 446 nm of the protein and DTNB solution. The protein concentration was 15 µM, and the DTNB reagent was 1 mM in 0.05 M phosphate buffer, pH 7.4 at 25°C. GSH was used to obtain a standard curve for free thiol concentration.

Figure 4  EPR spectra at 4.2 K of 1 mM deoxygenated Hb after NO gas was bubbled through the sample for 5 min. HbA0 (broken line); O-R-polyHbA0 (continuous line)

conditions, consistent with the reported lower reactivity of the thiol group in the T state [22]. The possibility, however, still exists that O-raffinose may directly or indirectly block these thiol groups.

EPR of HbNO

NO binds very strongly to the Fe(II) haems of haemoglobin [23]. Figure 4 shows the EPR spectra of HbNO for HbA0 and for O-R-polyHbA0. The three lines resolved on the high field side of the HbNO signal are attributed to nitrogen hyperfine lines resulting from the interaction of the nuclear spin on the nitrogen of NO with the unpaired electrons [24]. It has been shown [25,26] that when human HbA0 is in the T state the binding of NO to Fe(II) of the α chain causes the covalent bond between Fe(II) and the Nε of the proximal histidine to be broken, improving the resolution of the nitrogen hyperfine lines. In the R state, as found when NO binds to all the haems of HbA0, the hyperfine lines are not detected at all. The intensity of these hyperfine lines depends on the fraction of the bound NO on the α chain. In our sample, NO is binding to both α and β Fe(II) chains, unlike other studies of T-state haemoglobin where the low level of NO used binds exclusively to the α chains resulting in even greater resolution of these hyperfine lines. However, this result provides additional confirmation that O-R-polyHbA0 remains in the T state even when ligands are bound.

EPR of the metHb low-spin bis-histidine complexes

The low temperature EPR studies on metHb provide insights into the globin configuration and dynamics on the distal side of the haem. MetHb binds a water molecule at low and neutral pH. Water is a weak ligand producing a high-spin complex with \( g = 6 \) and \( g = 2 \). It has, however, been reported [27] that a low-spin complex is also observed at neutral pH (complex B), which involves the coordination of the distal histidine to the oxidized iron with the water molecule still in the haem pocket. Incubation of the sample at low temperature (in the region of \( -20°C \) to \( -80°C \)) results in the displacement of the water molecule by the distal histidine and the formation of a more stable bis-histidine complex (complex C).

The EPR spectra of complex B and complex C of HbA0 and O-R-polyHbA0 samples immediately frozen and refrozen after 18 min incubation at 235 K are shown in Figures 5(A) and 5(B). While complex B is clearly observed for HbA0, in both samples (Figure 5A), very little of it is observed in the immediately frozen sample of O-R-PolyHbA0 with almost none of it remaining after incubation (Figure 5B). The relative instability of complex B seen in Figure 5 for O-R-PolyHbA0 has previously been reported for deoxyhaemoglobin [28]. This comparison supports the contention that O-R-polyHbA0 is in the T state even when ligands are bound.

Reaction of nitrite with deoxyhaemoglobin

Nitrite reacts with deoxyhaemoglobin resulting in the formation of metHb and NO [29]. This reaction occurs only with deoxygenated

Figure 5  EPR spectra at 4.2 K of the small percentage of low-spin metHb present in 1 mM oxyhaemoglobin samples

Before incubation at 238 K (continuous line); after incubation at 238 K for 30 min (broken line). (A) HbA0; (B) O-R-polyHbA0.
haemoglobin chains. It is a multistep reaction [30], with the nitrite bound to deoxyhaemoglobin first converted to NO$^+$ bound to Fe(II). This complex is in equilibrium with Hb(III)NO with the eventual transfer of the NO from Fe(III) to another Fe(II) chain resulting in the formation of Fe(II)NO. Since this reaction does not take place with oxyhaemoglobin, it was not used to distinguish between haemoglobin in the R state and the T state. However, the steps in this reaction are very sensitive to the conformation of haemoglobin providing a probe for alterations in the T-state conformation.

The chemiluminescence determination shown in Figure 6 does not measure free nitrite or nitrosothiol, but under conditions of the assay in acetic acid can measure other haem-associated species present [30]. These include Hb(II)NO, Hb(II)NO$^+$, Hb(III)NO and Hb(III)NO. At the concentration of 20 $\mu$M nitrite and 100 $\mu$M haemoglobin, most of the signal detected in Figure 6 does not come from the stable Hb(II)NO, even after 60 min.

As shown in Figure 6 for HbA$_0$, O-R-polyHbA$_0$, and HbA$_0$ with IHP added, the chemiluminescence results indicate a biphasic reaction with an initial rapid phase followed by a slow phase. The initial very rapid reaction presumably involves the initial binding of nitrite and the second slower phase involves the formation of the intermediate which is represented by Hb(II)NO$^+$ ↔ Hb(II)NO [30]. The addition of IHP to deoxyhaemoglobin decreases the magnitude of the rapid phase and slows down the slow phase. O-R-polyHbA retains the same magnitude for the rapid phase as HbA$_0$ without IHP, but results in a rate for the slow phase, which is almost as slow as that of HbA$_0$ with IHP added. These changes can be attributed to the effect of modifying the T state on the binding of nitrite and formation of the intermediate, which is the initial nitrite reduced species.

**DISCUSSION**

In haemoglobin, which undergoes both tertiary and quaternary conformational changes as oxygen is bound, it is very difficult to fully explain how the oxygen affinity is regulated by these conformational changes. There has, therefore, been a great deal of interest in studying the properties of haemoglobin locked in either the T or R quaternary states, including a number of studies of abnormal haemoglobins or chemically modified haemoglobins, which remain in the R state even when oxygen is removed, and bind oxygen non-cooperatively with a high oxygen affinity [31,32].

A number of mutant haemoglobins with increased stability of the T state have also been studied. Some relevant examples are haemoglobin Kansas ($\alpha_2\beta_2$Asn-102 → Thr) [33], haemoglobin Presbyterian ($\beta$Asn-108 → Lys) and haemoglobin Yoshizuka ($\beta$Asn-108 → Asp) [34,35]. There have also been a number of re-combinant haemoglobins [36] that have increased stability of the T state. However, these mutants do not generally remain locked in the T state without any conformational changes produced by ligand binding.

Recent attempts to study T-state haemoglobin have, therefore, focused on methods to lock the protein in the T state even after ligands are bound. Studies have thus been performed with deoxyhaemoglobin crystallized from polyethylene glycol solutions. The external forces of the crystal inhibit the structural transition in the haemoglobin molecule [4] and the molecule stays locked in the T state. Studies have also been performed by encapsulation of deoxyhaemoglobin in wet porous silica gels. These gels are, however, only stable over a narrow pH and temperature range, and dramatically slow down, but do not completely prevent, the quaternary conformational changes.

Cross-linking of haemoglobin with O-raffinose not only stabilizes the tetrameric structure of the deoxyhaemoglobin (T state) by covalently cross-linking the two $\alpha\beta$ dimers but also oligomerizes the stabilized tetramer to increase its half-life in circulation. These modifications produced profound effects on oxygen affinity and cooperativity, which indicate that the haemoglobin is locked in the T state, providing an opportunity to explore functional properties of T-state haemoglobin in solution.

**Evidence of T state**

The non-cooperative oxygenation curve with $h = 1.0$ and a low apparent oxygen affinity with a predicted $p$50 of 50.9 (Figure 1, Table 1) is strong evidence for a molecule locked in the T state. Even though incomplete saturation would not preclude switching to R state at higher levels of oxygenation, the $h = 1$ through 70% oxygenation without any indication for an upward curvature, strongly suggests that the conformation stays locked into the T state. A shift in conformation at very high oxygen pressures would, however, not eliminate the value of studying a T-state haemoglobin at atmospheric oxygen pressures. The higher oxygen ‘off’ rate for O-R-polyHbA$_0$ is also consistent with haemoglobin in the T state at atmospheric oxygen pressures.

The activation enthalpy calculated from the binding of oxygen to normal haemoglobin includes both the enthalpy for binding oxygen and the enthalpy associated with the quaternary conformational change, which occurs during the binding of O$_2$. The 3.3 kcal decrease in enthalpy for O-R-polyHbA$_0$ is consistent with the haemoglobin remaining in the T-quaternary conformation during ligand binding. It has, thus, been reported that the enthalpy for the T to R quaternary conversion is 3–3.5 kcal/mole haem [37].

The ligand binding studies indicate that O-R-polyHbA$_0$ has the low oxygen affinity of T-state haemoglobin and does not undergo an increase in oxygen affinity during oxygen binding. However to determine that it is actually stabilized in the T state, we have investigated properties of this haemoglobin other than those directly related to ligand binding, which are known to depend on the quaternary conformation. These include changes that are
affected by the salt bridges at the interface as well as changes in the region of the haem on both the distal and proximal sides of the haem.

The Bohr effect is the change in the oxygen affinity with pH reflected in the lowest oxygen affinity in the region between pH 6 and pH 7 and an increase in oxygen affinity at both acidic and alkaline pH. These changes in oxygen affinity are attributed to changes in the $pK_a$ during oxygenation for a number of groups in the protein. These include the imidazoles of surface histidyl residues and the terminal amino acid residues [38]. The change in $pK_a$ is attributed to a change in conformation in the region of the group. The disruption of salt bridges at the interfaces during oxygenation has been shown to contribute to the Bohr effect. The smaller alkaline Bohr effect shown in Figure 2 is thus consistent with the salt bridges remaining intact during oxygenation.

The formation of bis-histidine complexes involving the binding of the N-terminus of distal histidine to the Fe(III) of the oxidized haem is sensitive to both structural changes as well as flexibility in the distal haem pocket [39,40]. Because the distal histidine is more than 4 Å away, low spin complexes associated with the distal histidine were originally attributed to structural changes which take place during denaturation of the haemoglobin [41]. It was subsequently shown [40] that dynamic fluctuations in the ligand pocket result in the approach of the histidine close enough to form a bond. At low temperature these fluctuations result in the formation of a complex with the distal histidine even for Fe(II) haemoglobin. The freeze-quenching studies on metHb [27] indicate that even at room temperature a complex of Fe(III) with the distal haem pocket [39,40]. Because the distal histidine is more than 4 Å away, low spin complexes associated with the distal histidine were originally attributed to structural changes which take place during denaturation of the haemoglobin [41]. It was subsequently shown [40] that dynamic fluctuations in the ligand pocket result in the approach of the histidine close enough to form a bond. At low temperature these fluctuations result in the formation of a complex with the distal histidine even for Fe(II) haemoglobin. The freeze-quenching studies on metHb [27] indicate that even at room temperature a complex of Fe(III) with the distal histidine is present. This complex with a $g_i$ corresponding to band B in Figure 5(A) was attributed to a seven-coordinated complex with both water and the distal histidine bound to the haem iron. The instability of this complex for O-R-polyHbA$_0$, (Figure 5) reflect subtle changes in the configuration of the distal pocket originating from structural and/or dynamic perturbations. The analogous decreased stability of the B-complex for the metHb chains of deoxyhaemoglobin in the T state relative to those of oxyhaemoglobin in the R state [28] is consistent with O-R-polyHbA$_0$ being in the T state even when oxygenated.

Reversible alterations in the distribution of bis-histidine complexes during low temperature incubation (in the region of $-20^\circ$C to $-80^\circ$C) [27,39,40] are a direct indication of protein fluctuations. The stabilization of complex C for O-R-polyHbA$_0$, thus indicates that the modification stabilizes the T state without preventing haem pocket fluctuations, which occur in the T state. In the crystal, it would be expected that even these fluctuations would be impeded.

In the T state the iron is displaced towards the proximal histidine resulting in appreciably weaker binding of ligands. NO binds very strongly to the Fe(II) haems, tending to pull the iron back into the haem plane. In the $\alpha$ chains this binding in the T state has been reported to rupture the bond with the proximal histidine [25,26]. The resultant increased nitrogen hyperfine lines produces a triplet signal used as a marker of the T-state quaternary conformation in most haemoglobins. The triplet signal observed for O-R-polyHbA$_0$, (Figure 4) in the presence of oxygen demonstrates the enhanced stability of the T state.

The quaternary conformation changes the environment of the $\beta$Cys-93 residue on the proximal side of the haem. In the R state it is exposed to the solvent and reacts with sulphhydril reagents [3,42-44]. In the T state, solvent access to the cysteine is impeded by the salt bridges formed in deoxyhaemoglobin [3] and exogenous sulphhydril reagents react much more slowly. Our data clearly indicates that the accessibility of $\beta$Cys-93 is greatly diminished in the modified raffinose derivative even in the presence of oxygen.

### Multiple T state

Crystal structures of haemoglobin have established the conformation of the high affinity R state of haemoglobin and the low affinity T state. More recently the X-ray structure of a third quaternary R2 state [45] was elucidated. Although the X-ray structure of more than one T state has not been elucidated, there is a great deal of evidence for more than one T state. It should be pointed out, however, that the presence of a high affinity T state does not necessarily involve a different quaternary conformation. It can instead be explained by a generalization of the MWC-PSK model [1,37–46] where within the T quaternary state there are transitions of $T$ and $R$, high affinity and low affinity tertiary conformations, which alter the oxygen affinity.

An additional functional state was originally proposed to explain the effect of protons, 2,3-DPG, IHP and CO$_2$ [37,47] on the oxygenation of haemoglobin. Our data for the effect of IHP on the $k_w$ for the binding of carbon monoxide to deoxyhaemoglobin (Figure 3) is consistent with this proposal. Thus even though deoxyhaemoglobin is in the T state before the addition of IHP, the addition of IHP lowers the rate constant for binding CO to HbA$_0$ (Table 1). The properties of O-R-polyHbA$_0$, are not affected by IHP (Table 1, Figure 3). However, by comparing the properties of O-R-polyHbA$_0$, with those of normal haemoglobin both before and after the addition of IHP (Figure 3), additional insights into the nature of the conformation of the cross-linked haemoglobin are provided.

The CO 'k$_w$' is measured with deoxyhaemoglobin, which is already in the T state. The lower rate when IHP is added to normal haemoglobin indicates a shift within the T state to a lower affinity T state. The results with O-R-polyHbA$_0$, indicate a rate between that obtained for HbA$_0$, with and without IHP. Perhaps the clearest evidence comparing the properties of O-R-polyHbA$_0$, with those of deoxyHbA$_0$, with and without IHP, is indicated by the data on nitrite reduction (Figure 6). The rapid reaction reflects the initial haemoglobin complex formed and the slower reaction is due to the formation of a subsequent complex with haemoglobin. Both the extent of formation of the initial complex and the rate for the conversion to the second complex depends on the protein conformation. The results indicate a qualitative difference between IHP, which changes the magnitude of the initial reaction and also slows down the subsequent reaction, while O-R-polyHbA$_0$, has no effect on the initial reaction, but dramatically slows down the subsequent reaction.

A comparison of the oxygen affinity obtained for O-R-polyHbA$_0$, indicates an appreciably higher $pK_50$ than for crystallized T-state haemoglobin [48]. This discrepancy can be attributed to the constraints placed on the T-structure by the crystal lattice. However, it may also suggest that the crystal is trapped in a low affinity T state while the cross-linked haemoglobin is in a state with intermediate affinity.

With cross-linked haemoglobin in an intermediate T state, our results provide valuable insights into the nature of this intermediate state. The Hill coefficient 'h' equal to 1.0 indicates that we do not have a mixture of states, which would result in $h < 1$. Instead the crosslinked haemoglobin is trapped in an intermediate T state. The low temperature EPR data (Figure 5B), nevertheless, indicate that protein fluctuations occur within this intermediate T state. The finding that $h = 1$ also limits the possible differences in oxygen affinity for $\alpha$ and $\beta$ chains in the cross-linked state.

O-R-polyHbA$_0$, has been reported to contain a mixture of different oligomers of tetrameric haemoglobin [9]. While this raises the possibility of heterogeneity in terms of the ligand binding properties, the $h = 1$ is clear evidence that the oxygen binding properties of all of the forms present in our sample are quite similar.
(differences in oxygen affinities even with all non-cooperative agents would result in $h < 1$). This prediction is supported by recent studies performed in our laboratory (R. A. Boykins, Y. Jia, P. W. Buchler, R. Venable and A. I. Alayash, unpublished results). As part of our ongoing investigation into the origin of the functional abnormality in this heterogeneous compound we have fractionated $O$-R-polyHbA into 6 fractions using size exclusion chromatography. Initial assessment of the separated fractions appears to indicate a similarity among the isolated fractions in their oxygen equilibrium and kinetics parameters compared with the unfractonated parent compound, $O$-R-polyHbA.

**Physiological implications**

The properties of the haemoglobin derivative described herein are consistent with the protein lacking both cooperativity and sensitivity to allosteric modifiers. As a result this particular haemoglobin appears to be locked in the quaternary T state. In addition to these functional defects, $O$-R-polyHbA undergoes rapid autoxidation and oxidative side reactions, including the formation of rhombic haem [12]. It is difficult to correlate these in vitro characteristics to potential toxicity in vivo, it is however noted worthy that Hemolink$^T_M$, the commercial form of this haemoglobin has recently been withdrawn from further clinical development due to unexpected side effects in patients undergoing cardiac surgery [49].

The opinions and assertions contained herein are the scientific views of the authors (Y.J., F.W. and A. I. A.) and are not to be construed as policy of the United States Food and Drug Administration.

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Cross-linking of haemoglobin with O-raffinose


Received 13 April 2004/7 July 2004; accepted 10 August 2004
Published as BJ Immediate Publication 10 August 2004, DOI 10.1042/BJ20040612