Full Length Research Paper

Studies on the reactions of hydrogen peroxide with Sickle cell haemoglobin

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The reaction between hydrogen peroxide and myoglobin (or haemoglobin) ferric haem is a two-electron redox process, yet the stable final product is ferrylhaem, retaining only one oxidizing equivalent. The ferrylhaem produced from the reaction enhances oxidation-related toxicity associated with inflammation, ischemia and hemolytic disorders. The aim of this study was to compare sickle cell and normal adult haemoglobin reactions with H₂O₂ to elucidate the possible role of such reactions in the pathology of sickle cell anaemia. This study was carried out using UV-visible spectroscopy and HPLC. The UV-visible spectroscopy investigations show that the reactivity of ferric HbS with hydrogen peroxide shows identical and almost indistinguishable pattern to that of normal adult ferric HbA. The HPLC chromatographic pattern of hydrogen peroxide induced haem-protein cross link in sickle cell haemoglobin (HbS) is identical to that of normal adult haemoglobin (HbA). This probably underlines the fact no significant instability exist in the relationship between the sickle cell Hb haem pocket and its globin as a result of the mutation.

Key words: Sickle cell anaemia, sickle cell haemoglobin, hydrogen peroxide, haemoglobin, ferryl haemoglobin.

INTRODUCTION

Sickle cell disease is a haemoglobin structural defect in which a glutamic acid residue at the 6th position in the haemoglobin β-chains is replaced by valine. This change causes a drastic reduction in the solubility of the sickle haemoglobin (HbS) when deoxygenated. The deoxyhaemoglobin is unstable and tends to precipitate on the erythrocyte membrane, setting the scene for site-specific free-radical reactions that promote lipid peroxidation and damage to membrane proteins (Halliwell and Gutteridge, 2001). Iron may also be released to bind to the membrane to alter erythrocyte membrane structure and functions (Shalev and Hebbel, 1996). However, both normal haemoglobin (HbA) and sickle haemoglobin (HbS) are isomorphic having multiple identical functions and show only minor differences at the N-terminal end of their respective β-globin chains (Sheng et al., 1998). Although, HbS does manifest one dramatic behavioural difference in the form of an abnormal denaturation tendency that is derived from the mutant Hb’s hydrophobic surface substitution. Compared with normal red blood cells, sickle red blood cells have been reported to spontaneously generate approximately 2-fold greater extents of superoxide, H₂O₂ and hydroxyl radical (Hebbel et al., 1982). Furthermore, the faster haem loss from HbO₂S is attributed to accelerated auto-oxidation (HbO₂S→ferricHb) rather than to some other type of instability inherent in the relationship between the sickle haem pocket and its globin (Hebbel et al., 1988). However, it has been reported that reactive oxygen species formation is in fact not increased in sickle cell red cells and that the rates of consumption of nitric oxide (NO) by normal and sickle erythrocyte are similar, hence directing attention elsewhere in the search for the source for reactive oxygen species (Aslan et al., 2001). It has been estimated that about 3% of the haemoglobin present in human erythrocytes undergoes autooxidation every day to generate superoxide radical (O₂•⁻) and
ferricHb (Winterbourn, 1985). Hydrogen peroxide is produced from dismutation reaction of superoxide radical (\( \bullet O_2^- \)) catalysed by superoxide dismutase (1). The reaction of haem-proteins with \( \text{H}_2\text{O}_2 \) not only oxidizes the iron to the ferryl (Fe(IV)=O) state but also produces amino acid radical on the protein that is rapidly dissipated by poorly understood mechanism (Catalano et al., 1989). The tyrosine and tryptophan peroxyl radicals (Try/TyrOO•) have been identified (Gunther et al., 1995) during this reaction. Cross-links between the haem and protein can also occur (Catalano et al., 1989), in the reaction pathway between haem-proteins and hydrogen peroxide. Although the mechanism of formation of this haem-protein cross-linked species (Mb-H) involved a protein radical (possibly a tyrosine) and ferrylhaem, Reeder et al. (2002) has proposed that in addition to a protein-based radical, the protonated form of the oxoferrylhaem, known to be highly reactive and radical-like in nature, is required to initiate cross-linking. Formation of (Mb-H) is a specific marker for the peroxide reaction with haem-protein as it is stable both in vitro (Catalano et al., 1989) and in vivo (Moore et al., 1998). This in vivo marker can exacerbate the severity of oxidative damage due to its enhanced prooxidant and pseudoperoxidative activities (Moore et al., 1998). Investigation into the nature of ferryl species has shown that decay of the ferryl species was more rapid at acid pH values and ferryl reacted faster with reducing agents under acidic conditions (George and Irvine, 1951, 1952).

Considering the background given above regarding the ferryl species and since the footprint of free-radical mediated oxidative damage in sickle erythrocytes has been recognized for a number of years, hence the aim of this study was to compare sickle cell and normal adult haemoglobin reactions with \( \text{H}_2\text{O}_2 \) to elucidate the possible role of such reactions in the pathology of sickle cell anaemia. This study was carried out using UV-visible spectroscopy and HPLC.

**MATERIALS AND METHODS**

Potassium ferricyanide, sodium dithionite (sodium hydrosulfate) and hydrogen peroxide were purchased from Sigma-Aldrich Co (Poole, UK). HPLC grade acetonitrile and trifluoroacetic acid were purchased from Fisher Scientific (Loughborough, UK). Human blood (normal) was obtained from volunteer registered donor while the sickle cell blood was from the Department of Haematology at University College London Hospital (UCLH) London. The HbAA and HbSS genotypes were confirmed electrophoretically.

**Preparation of hydrogen peroxide solution**

The stock 30% w/w hydrogen peroxide of about 10 M was purchased from Sigma. Dilute solution of hydrogen peroxide were prepared fresh daily from the stock. Hydrogen peroxide solution was prepared by making appropriate dilution in appropriate buffer or deionised water and kept on ice. The exact concentration of hydrogen peroxide was determined spectrophotometrically by measuring the absorbance at 240 nm using a molar extinction coefficient of 40 M\(^{-1}\) cm\(^{-1}\) as described (Giulivi and Davies, 1994).

**Preparation of buffer solutions**

Most of the buffers used throughout this work were within the pH range of 5 to 11. The buffers used for pH 5 and 5.5 are sodium acetate buffers; this was prepared by making a solution of sodium acetate of the required molarity in deionised water and adjusting the pH with glacial acetic acid. The buffers used for pH 6 to 8.5 inclusive are mostly sodium phosphate buffers: These are prepared by making solutions of sodium dihydrogen phosphate and disodium hydrogen phosphate to the required molarity in deionised water. These were mixed together until the desired pH was attained. For pH 9 to 11 mostly sodium tetraborate buffer was used: it was prepared by making a solution of sodium tetraborate of the required molarity in deionised water and adjusting the pH upward with sodium hydroxide or downward with hydrochloric acid until the desired pH was attained. Other buffers were prepared such as a tris buffers etc in similar manner as was mentioned above depending on the requirement. However, most of the buffers prepared where necessary contain iron chelators such as EDTA or DTPA.

**Preparation of human haemoglobin (HbA and HbS)**

Human haemoglobins (the abnormal haemoglobin, sickle cell haemoglobin (HbS) from a sickle cell blood and normal adult haemoglobin (HbA) from adult normal blood) were isolated from their respective erythrocytes according to the method as previously described (Antonini and Brunori, 1971), with some modifications. The human blood sample (15 ml) was drawn into sodium citrate (1 - 2% final concentration). The blood was diluted with 30 ml of 1% sodium chloride solution and centrifuged at 2000 rpm at 10°C for 15 min. The supernatant containing plasma was removed and the cells re-suspended in 30 ml of sodium chloride solution (1%) followed by centrifugation at 2000 rpm 10°C for 15 min. This step was repeated three times. The washed red cells were lysed by the addition of 45 ml of cool distilled water (three times initial volume of blood) and left on ice for 15 min. Addition of water yields a mixture of haemoglobin and red cell fragment. The haemolysate was treated with saturated neutral ammonium sulphate.
solution. To every 80 ml of haemoglobin solution, 20 ml of saturated neutral ammonium sulphate was added and the solution again left on ice for 50 min. The mixture was then centrifuged at 12,000 rpm (approximately 17,000 g) at 4°C for 30 min. The red jelly-like precipitate was discarded while the supernatant containing essentially haemoglobin was dialysed for 72 h in 3 mM sodium phosphate buffer pH 7.4 at 4°C with 5 changes of buffer. Several buffer changes are required to remove the ammonium sulphate salt.

During the preparation, carbon monoxide was blown over the surface of, or gently bubbled through the blood/haemoglobin solution. Prior to the dialysis the haemoglobin solution was saturated with CO. This converted the HbO₂ to the carbon monoxide form with the aim of preventing auto-oxidation. The dialysed haemoglobin solution was again saturated with CO. To further purify the haemoglobin solution, catalase was removed from the haemoglobin solution by ion exchange chromatography on DEAE Sephadex A50 (Bonaventura, 1972). The pH of the haemoglobin solution was adjusted to pH 6.7 with dilute acetic acid before being applied to a column, previously equilibrated with 5 mM sodium phosphate buffer pH 6.7. At this pH value, HbCO which has a pl of 7.2 passes down the column while catalase with a pl of 5.8 is retained.

The purity of the haemoglobin prepared from the method previously described was determined by HPLC. The HPLC chromatogram measured at 280 nm (Figure 5) show the haemoglobin and its subunits are pure and free from proteins including antioxidant enzymes (e.g. catalase) that interfere with the reactions under studies. The alpha and beta chains of the haemoglobin are separated and remain intact as does the haem group.

The reverse phase (HPLC) measurements

The reverse phase HPLC was adapted from the method as previously described (Bonaventura, 1972). Samples were analyzed on an HP 1100 HPLC fitted with a diode array spectrophotometer. The column used was a Zorbax Stable Bond 300 C3 250 mm x 4.6 mm guard column. Solvent were (A) 0.1% trifluoroacetic acid (TFA) and (B) acetonitrile containing 0.1% TFA. The gradient was initially 35% solvent B, stable for 10 min, increasing to 37% solvent B over 5 minute. This was increased to 40 % solvent B over 1 min and then to 43% solvent B over 10 minutes. The flow rate was 1 mL min⁻¹ and temperature was 25°C.

Preparation of ferric haemoglobin (HbS and HbA)

Ferric HbS was prepared from sickle cell haemoglobin isolated and purified by the method previously described above. The haemoglobin stored as HbCO was converted to HbO₂ by directly illuminating with continuous intense white light and simultaneously passing a stream of oxygen over the solution stored on ice. The ferric HbS was prepared by addition of slight excess (about 10%) stoichiometric amount of potassium ferricyanide to the HbO₂ solution. The conversion to ferric form was immediately detected by change in colour of the solution from bright red to brown. Thereafter, a gel filtration chromatography was performed by passing the mixture through a Sephadex G-25 column equilibrated with 0.1M potassium phosphate, pH 7.0, to remove residual ferricyanide and ferrocyanide. The concentration of ferric HbS was determined using an absorbance extinction coefficient = 11 mM⁻¹ cm⁻¹ at 541 nm in 0.1 M potassium phosphate, pH 7.0 (Antonini and Brunori, 1971).

In a similar manner, normal ferric haemoglobin was prepared from normal adult haemoglobin (HbA) isolated and purified as for ferric HbS method described above.

Experimentals

Titration of ferric HbS with hydrogen peroxide

Ferric sickle cell Hb was titrated with H₂O₂ according to the method described previously for haem-protein titration with ligands. The reaction was performed in 0.1 M sodium phosphate pH 8.0 buffer containing (100 µM) DTPA and the temperature kept at 30°C throughout the experiment. A small volume of 10 µL of 2.5 mM HbO₂ was added to 10 µM ferric sickle cell Hb spectra recorded after 5 min. Additions of 10 µL were repeated until no further absorption changes were observed and complete saturation was obtained. For each addition of H₂O₂ the reaction was allowed for equilibration for 5 min. The absorption spectrum recorded using a Varian Cary 5E spectrophotometer. Spectra were recorded before and after each addition of H₂O₂ (~2.5 µM aliquots) to the sickle Hb. The titration was terminated at 50 µM H₂O₂. The absorbance decrease at 406 nm due to depletion of ferric HbS, and the absorbance increase at 425 nm due to ferryl HbS formation. A pH 8.0 buffer solution was used because at this pH the ferri Hb formed tends to be more stable. Likewise, normal ferricHb was titrated with H₂O₂ as previously described for ferric sickle cell Hbabove.

HPLC measurements for reactions of H₂O₂ with sickle cell haemoglobin (HbS) or normal HbA

The HPLC injector program was used to examine the initial time course of formation of oxidatively modified haemoglobin. Ferric HbS (8 mL, 320 mM; 100 mM final) was drawn into the HPLC injector module after 1 mL of 25 mM sodium acetate buffer, pH 5.0 containing 100 mM DTPA. The injector needle was washed, and a further 13 mL of buffer was drawn to the injector. The injector
Figure 1. Sickle cell ferric Hb spectral changes induced by H$_2$O$_2$ titration.

RESULTS

Sickle cell haemoglobin spectral changes induced by H$_2$O$_2$ titration

Figure 1 shows the sickle cell ferric Hb spectra changes before and after titration with known concentrations of H$_2$O$_2$. The difference spectrum of the haemoglobin after the titration was terminated and before the addition of H$_2$O$_2$ shows significant changes in both the visible and Soret region of the spectrum. These changes resulted in a decrease in the ferric HbSSoret peak at 406 nm and the formation of a shoulder at 427 nm. These changes in the absorption spectrum of sickle cell haemoglobin are similar to those reported for normal haemoglobin (HbA) reaction with H$_2$O$_2$ (Figure 2) and was understood as a redox transition from ferric to its ferryl form which lack a shoulder at 630 nm. Figure 3 show that the sickle cell Hb/H$_2$O$_2$ reaction stoichiometry is 1:1 as expected as previously described in equation 1 (the radical cation having migrated away). Similarly, ferricHbA/H$_2$O$_2$ reaction stoichiometry is 1:1 (Figure 4). The absence of catalase in the sickle cell haemoglobin used for the entire study was demonstrated as shown in the HPLC chromatogram in Figure 5. This reaction below

Fe(III) + H$_2$O$_2$ $\rightarrow$ Fe$^{IV}$=O$_2^-$ + H$_2$O  \hspace{1cm} (1)

would be perturbed if catalase, which directly catalyses decomposition of H$_2$O$_2$ to ground state oxygen, were present.

Sickle cell ferricHb (10 µM) was titrated with H$_2$O$_2$ in sodium phosphate, 0.1 M, pH 8.0 at 30°C. At pH 8 the ferryl species is stable. Spectrum was recorded before and after additions of a small volume of 10 µL of 2.5 µM H$_2$O$_2$ to the ferric HbS at intervals of 5 min to allow for equilibration. The additions were repeated until no further absorbance changes were observed and complete saturation was obtained. For each addition of H$_2$O$_2$ the reaction was allowed for equilibration for 5 min. In subsets: (A) Difference spectrum of the final haemoglobin after the last titration with H$_2$O$_2$ and ferric HbS before the addition of H$_2$O$_2$. (B) 4 fold magnification of spectral changes in the visible region.

FerricHb (A) (10µM) was titrated with H$_2$O$_2$ in sodium phosphate, 0.1M, pH 8.0 at 30°C. At pH 8 the ferryl species is stable. Spectrum was recorded before and
Figure 2. FerricHb(A) spectral changes induced by H$_2$O$_2$ titration.
Figure 3. Titration of 10 µM ferric HbS with known concentrations of H₂O₂ in 0.1 M sodium phosphate pH 8.0.
Figure 4. Titration of 10µM ferric HbA with known concentrations of H$_2$O$_2$ in 0.1 M sodium phosphate pH 8.0.

Figure 5. HPLC chromatogram of (100µM) normal haemoglobin (HbA) and (100µM) sickle cell haemoglobin (HbS) without addition of H$_2$O$_2$. 
Haem B
oxidatively modified haems

Figure 6. HPLC Chromatogram of HbA (100µM) and HbS (100µM) after their reaction each with H2O2 (400 µM).

The temperature was kept at 30°C throughout the experiment. At 10 µM H2O2 concentration, one molecule of hydrogen peroxide was required to oxidise one molecule of ferric HbS to form a molecule of ferrylHb. Beyond 30 µM H2O2 concentration, the reactions of H2O2 with ferric HbS show some evidence of haem decay.

The temperature was kept at 30°C throughout the experiment. At 10 µM H2O2 concentration, one molecule of hydrogen peroxide was required to oxidise one molecule of ferric HbS to form a molecule of ferrylHb. Beyond 35 µM H2O2 concentration, the reactions of H2O2 with ferric HbS show some evidence of haem decay.

HPLC of sickle cell haemoglobin reaction with H2O2

The reverse-phase HPLC method was used for studying the reaction between HbS and hydrogen peroxide. This method separates the components of the reaction into haem-protein cross-linked species, unmodified “free” haem (i.e. not covalently bound to protein) and oxidatively modified “free” haems. Figure 5 show the chromatograms of normal ferric HbA and sickle cell ferric HbS measured at 280 and 400 nm, where due to the acid solvent conditions, the unmodified haem (iron protoporphyrin IX, haem B) and the alpha and beta chains were separated and remained intact at 280 nm. The haem B components for both sickle cell and normal haemoglobin have elution time of 15.14 min, while the alpha chains for both sickle cell and normal haemoglobin were eluted at 24.28 min. The beta chain for normal haemoglobin has elution time of 22.74 min. While that of sickle cell was eluted at 22.92 min. The chromatograms at 280 nm for both proteins show that the absorbance peak of beta chain was more than that of alpha chain. This indicates clearly that the beta chain has more aromatic amino acids than alpha chain. The chromatogram measured at 280 nm for the products of the reaction of H2O2 with sickle cell ferric Hb and normal ferric Hb in the ratio of 4:1 is shown in Figure 6. This Figure shows the separation of the components into oxidatively modified free haem (7 - 14 min elusion time), unmodified haem (15.1 min elusion time), unmodified protein species (alpha and beta chains) (21.3-27.1 min elusion time) and probably a “new protein species” (28.9 min elusion time). However, examination of the chromatogram at 400 nm for the products (Figure 7) shown that actually not a “new protein species” that seen at 280 nm but the presence of
Figure 7. HPLC chromatogram of normal and sickle cell haemoglobin after their reactions with hydrogen peroxide measured at 400 nm.

haem-protein cross-linked protein species that are very distinct with a broadened band (21 - 29.5 min elusion time) that is more apparent probably due to higher concentrations of hydrogen peroxide used. The cross-linked protein species measured at 400 nm is typical of tryptophan and tyrosine bands, indicating the present of these proteins, thus confirming these as haem-proteins cross linked species. The haem-protein cross-linked species are heterogenic in nature and this may arise from oxidative damage to the protein affecting the retention time of haem-protein cross-linked species on the column but may also reflect the different ways of cross-linking the haem to the protein. The chromatogram also show increase amounts of oxidatively damaged haem.

The mechanism of haem-protein cross-linking in myoglobin reported, Reeder et al. (2002), explained why cross-linking occurs at a low pH values. This mechanism indicates that cross-linking will only occur if the protein radical and protonated oxoferryl species are present. At pH values above 6 the ferryl species can be formed and is relatively stable, yet no significant cross-linking occurs.

A simple equation describing the react of ferric HbS (Hb-Fe$^{3+}$) reacts with H$_2$O$_2$ to form the ferryl species and a protein cation radical species ($P^\bullet^\ast$). As described previously, the radical partially resides on amino acid residue (possibly tyrosine) (Catalano et al., 1989).

The HPLC chromatograms were measured at 280 and 400 nm for both HbA and HbS. The alpha and beta chains of the haemoglobins and also their haem groups remain intact. (See HPLC measurement conditions).

The HPLC Chromatogram for ferryl HbA and ferryl HbS were measured at 280 nm. The haem and oxidation products from the reactions in 25 mM sodium acetate at pH 5.0 indicate the present of new protein. (See HPLC measurement conditions).

Haem and protein oxidation products from the reactions of H$_2$O$_2$ (400µM) with ferric HbA, and ferric HbS (100µM) at 400nm show haem-protein cross link. (See HPLC measurement conditions).

Discussion

Like with normal HbA, the sickle cell Hb/H$_2$O$_2$ reaction stoichiometry is 1:1 and ferrylHbS as a product was observed. This is consistent with the suggestions on the reaction between ferric myoglobin and hydrogen peroxide in which ferrylMb generated (George and Irvine, 1952). This probably underlines the fact no significant instability exist in the relationship between the sickle cell Hb haem.


