Kinetico-mechanistic Studies on Methemoglobin Generation by Biologically Active Thiosemicarbazone Iron(III) Complexes

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Abstract

The oxidation of human oxyhemoglobin (HbO₂) to methemoglobin (methHb) is an undesirable side effect identified in some promising thiosemicarbazone anti-cancer drugs. This is attributable to oxidation reactions driven by Fe^{III} complexes of these drugs formed *in vivo*. In this work the Fe^{III} complexes of selected 2-benzoylpyridine thiosemicarbazones (HBpT), 2-acetylpyridine thiosemicarbazones (HApT), and the clinically trialled thiosemicarbazone, Triapine[®] (3-amino-2-pyridinecarboxaldehyde thiosemicarbazone, H3-AP), have been studied. This was achieved by time-resolved UV-Visible absorption spectroscopy and the sequential oxidation of the α - and β - chains of HbO₂ at distinctly different rates has been identified. A key structural element, namely a terminal -NH₂ group on the thiosemicarbazone moiety, was found to be an important common feature of the most active HbO₂ oxidising complexes that were investigated. Therefore, these studies indicate that an unsubstituted -NH₂ moiety at the terminus of the thiosemicarbazone group should be avoided in the design of future compounds from this class.

Keywords: thiosemicarbazone, iron, haemoglobin, methemoglobin

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Introduction

Hemoglobin (Hb) is an $\alpha_2\beta_2$ tetrameric hemoprotein essential for the transport of dioxygen in vertebrates [1]. Oxyhemoglobin (HbO_2) is the active form comprising four heme prosthetic groups each ligated by a single dioxygen molecule. The mechanism by which dioxygen is bound and released is relatively well understood [2]. The four hemes do not act independently, but rather the binding of dioxygen is cooperative and each O_2 ligand is bound successively more tightly coupled with protein conformational changes [2]. A key feature is that the dioxygen binding affinity of HbO_2 is acutely sensitive to the oxidation state of the Fe ion within each heme prosthetic group. Oxidation of the (formally) ferrous heme to ferric heme leads to complete loss of dioxygen affinity. This can be triggered *in vivo* by conditions of oxidative stress or specifically by reaction with small molecular weight oxidants [3, 4].

Under physiological conditions (*i.e.* at pH 7.4 and when Hb is saturated with dioxygen), auto-oxidation of HbO_2 is very slow [5, 6]. The fully (auto)oxidised and inactive protein methemoglobin (metHb) constitutes 1-2% of the protein in erythrocytes (red blood cells) [7]. The enzyme methemoglobin reductase (NADH-cytochrome b_5 reductase) [8] rescues this inactive form by reducing metHb to HbO_2 *in vivo* [1]. Interestingly, the rates at which the α - and β -chains of HbO_2 undergo (auto)oxidation are not the same [9]. Investigations of isolated α -chains of HbO_2 revealed that autooxidation is significantly faster ($7 \times 10^{-6} \text{ s}^{-1}$) than the isolated β -chains ($2.5 \times 10^{-6} \text{ s}^{-1}$). Similar findings have emerged in investigations of the HbO_2 tetramer [9].

Outer sphere electron transfer reactions between synthetic complexes and HbO_2 , and its related monomeric protein myoglobin, have been extensively investigated [3, 10-17]. Given the environment of the heme prosthetic group, only an outer sphere mechanism is plausible and indeed this has been established [13, 14]. The four hemes are partially solvent exposed and small molecular weight oxidants can approach the heme edge and accept electrons from this prosthetic group [3, 10, 18].

The study of hemoglobin oxidation has relevance to the known condition methemoglobinemia, where excessive oxidation of HbO₂ to metHb occurs and this leads to tissue hypoxia [4, 7, 19]. Methemoglobinemia is a known side effect of the anti-cancer agent, 3-amino-2-pyridinecarboxaldehyde thiosemicarbazone (**H3-AP** or Triapine[®], Chart 1) [20], which has been examined in numerous Phase I and II trials [21-28]. This deleterious property has also been identified in some related thiosemicarbazones from the di-2-pyridylketone thiosemicarbazone (**HDpT**) family (Chart 1) [4] and a molecular understanding of this side effect is an important goal in order to better tune the biological properties of these compounds. A common feature of the compounds in Chart 1 is that they are avid Fe chelators that always coordinate as tridentate *NNS* chelators [29-32]. It has emerged that their favourable anti-cancer properties are, in part, due to their ability to coordinate labile intracellular Fe *in vivo* [33]. In addition the Fe complexes of the **HDpT**, **HBpT** and **HApT** series (and Triapine[®]) are redox active and may readily cycle between their Fe^{II} and Fe^{III} oxidation states, which leads to hydroxyl radical formation (Fenton chemistry) and cytotoxicity [34].

The redox potentials of Fe complexes from the **HDpT** [29] series can be “tuned” by substitutions on the terminal N⁴-atom of the thiosemicarbazone (Chart 1) or by moving to the **HBpT** [30] and **HApT** [35] series based on the ketone precursors 2-benzoylpyridine and 2-acetylpyridine, respectively. The inductive effects of these substituents are communicated to the Fe ion in a predictable way [35]. It also has been shown that [Fe^{III}(Dp44mT)₂]⁺ and [Fe^{III}(Bp4eT)₂]⁺ induce HbO₂ oxidation on intact human erythrocytes, erythrocyte lysates and purified oxyhemoglobin [4]. In an initial investigation [17], we revealed some interesting structural features that led to some members of the Fe^{III}-**HDpT** complex family being more active than others toward HbO₂ oxidation. Against this background, herein we report a kinetic-mechanistic study on the ability of the Fe^{III} complexes of the **HBpT** and **HApT** series of thiosemicarbazone ligands (Chart 1) to promote metHb generation *in vitro* as a model for the oxidative stress process *in vivo*. We have also included the Fe complex of Triapine[®] in this study because of its known ability to stimulate the production of metHb *in vivo* [26, 36].

Chart 1 Thiosemicarbazone ligands involved in this work (the labile proton designated by the H-prefix of each ligand is shown in bold type and the N⁴-position is indicated for **HDpT**). The Fe complexation mode is shown for DpT⁻ (anion) and is representative of all ligands. The abbreviation follows the sequence of the parent ketone (Dp, Bp or Ap for dipyridyl, benzoylpyridine or acetylpyridine) followed by the N⁴ substituents: e = ethyl; m = methyl; 44m = dimethyl).

Experimental

Chemicals and reagents

The 2-benzoylpyridine thiosemicarbazones **HBpT**, **HBp4eT**, **HBp4mT** and **HBp44mT** as well as their Fe^{III} complexes, $[\text{Fe}^{\text{III}}(\text{Bp}xx\text{T})_2]\text{ClO}_4$, were prepared as described in the literature [30]. The 2-acetylpyridinepyridine thiosemicarbazones **HApT**, **HAp4mT** and **HAp44mT** and their Fe^{III} complexes, $[\text{Fe}^{\text{III}}(\text{Ap}xx\text{T})_2]\text{ClO}_4$, were also prepared following established procedures [35]. 3-aminopyridine-2-thiosemicarbazone (**H3-AP**, Triapine[®]) was prepared as described [20], as was its Fe^{III} complex [37]. All other reagents and solvents were obtained commercially and used without further purification.

Purification of commercial haemoglobin

Solutions of HbO_2 were prepared afresh before every set of experiments. Human hemoglobin (Sigma) was purified by dissolving *ca.* 40 mg of protein in 1.5 mL of Hank's Balanced Saline Solution (HBSS) then reducing the protein with excess sodium dithionite (*ca.* 1 mg). A pure solution of HbO_2 was obtained after gel filtration through a HBSS-equilibrated PD-10 column (Pharmacia; prepacked 8.3 mL of Sephadex G-25). This stock solution was used for all subsequent measurements. The concentrations of the HbO_2 solutions were determined from the extinction coefficient of the protein at 542 nm [38].

Kinetic studies

Kinetic measurements of the reaction between human HbO_2 (Sigma) and the corresponding Fe^{III} complex were carried out with at least a 10-fold ($15\text{--}60 \times 10^{-6}$ M) molar excess over HbO_2 concentration which was *ca.* 0.75×10^{-6} M tetramer (3×10^{-6} M total heme). All experiments were carried out at 25 °C. Stock solutions of the $[\text{Fe}^{\text{III}}(\text{L})_2]^+$ complexes were 1 mM in MeOH (due to poor aqueous solubility) and the samples for kinetic measurements were prepared by substantial dilution of this stock solution in aqueous buffer (final composition of the solution was < 1% MeOH). The Fe complex concentration range was limited to less than an order of magnitude above the HbO_2

concentration due to the strong absorbance of the Fe thiosemicarbazone complexes which obscured any meaningful changes in the HbO₂ spectrum at higher concentrations.

Reactions in the seconds timescale were monitored on an Applied Photophysics SX20 MV Stopped-flow instrument with photo-diode array detection, while reactions in timescales of minutes were monitored using an Agilent 8453 diode array UV-Visible absorption spectrophotometer fitted with a multicell transport. In all cases, the full time resolved spectra were analysed by ReactLab Kinetics [39]. The general kinetic technique has been previously described [40]. Experiments were set up in such a way that the spectral changes were consistent with a single step first order ($A \rightarrow B$) reaction; the data were typically fit to a single exponential change. Nevertheless, for slower reactions initiated by manual mixing, a consecutive ($A \rightarrow B \rightarrow C$) first order reaction process could be simulated using stopped flow data for the initial rapid reaction to produce an overall consistent profile comprising both steps. Table S1 collects all the values of the observed rate constants measured for the different systems.

Results

Biological Relevance

Given our previous knowledge on the oxidation reactions of HbO₂ by the **HDpT** series of Fe^{III} complexes [4, 17] and that the **HDpT** series may be altered by choice of a different parent ketone, tuning the reactivity toward HbO₂ can be easily achieved (Chart 1). Using 2-benzoylpyridine (instead of di-2-pyridyl ketone) a phenyl ring may be introduced at the imine carbon (instead of a non-coordinating pyridyl ring), generating the 2-benzoylpyridine thiosemicarbazone series (**HBpT**) [30]. Many of these chelators were found to exhibit more potent anti-cancer activity than the corresponding **HDpT** series [30] and showed high selectivity for cancer cells, namely IC₅₀ values < 1500-fold relative to normal cells. Similarly, replacing the electron withdrawing (non-coordinating) pyridyl ring in the **HDpT** series, with an electron donating methyl group, results in the 2-acetylpyridine thiosemicarbazone analogues (**HApT**), that possess even greater anti-proliferative activity (IC₅₀ = 0.001-0.002 μ M) against cancer cells than the most effective **HDpT** and **HBpT** analogues [35]. In both cases, these chelators bind iron by *NNS* donor atoms, forming highly stable Fe^{II} and Fe^{III} complexes with a 2:1 ligand to Fe ratio [30].

Kinetic measurements

The kinetics of HbO₂ oxidation by the Fe^{III} complexes of the **HBpT** series (**HBpT**, **HBp4eT**, **HBp4mT** and **HBp44mT**), **HApT** series (**HApT**, **HAp4mT** and **HAp44mT**), and Triapine[®] (**H3-AP**) (Chart 1) have been examined under the same conditions as the **HDpT** series [17]. Analysis of the time-resolved spectral changes revealed a two-step process on different time-scales; no more than two steps were identified. This fact is consistent with the α - and β -chains of HbO₂ reacting at different rates, in line with known auto-oxidation kinetics [9]. Given the fact that each $\alpha\beta$ -HbO₂ dimer is in equilibrium with its tetramer, we can consider $\alpha\beta$ -HbO₂ (within its tetramer or isolated dimer) as the reactive species in all cases. Reaction of the two heme groups of the $\alpha\beta$ dimer with two Fe^{III} thiosemicarbazone complexes (**HBpT** and **HApT** series) is required to reach fully oxidised metHb

(Equation 1). Nevertheless, this oxidation reaction (Equation 1) is complicated, involving necessarily the oxy/deoxy-hemoglobin equilibria. In fact, the process involves deoxyhemoglobin as the reactive intermediate (not shown here), as found in the previous investigation of the **HDpT** series [17].

Figure 1 illustrates representative time-resolved UV-Vis spectrophotometric changes in the 400-800 nm wavelength range, consistent (see above) with sequential oxidation of the two heme groups in the $\alpha\beta$ HbO₂ moiety. The results mirror those of the **HDpT** series [17], which show a decrease in intensity of the HbO₂ bands at 577 and 539 nm with an increase in intensity and hypsochromic shift of the Soret band at 404 nm which is due to the conversion of HbO₂ into metHb [38]. Furthermore, the increase in absorbance of the broad maximum around 620 nm on the short timescale experiment (Figure 1A) can be attributed to accumulation of $[\text{Fe}^{\text{II}}(\text{ApT})_2]$ which absorbs in this region. On the longer timescale (Figure 1B) the Fe^{II} complex is reoxidised by dioxygen in solution (Equation 2).

Figure 1. (A) Fast spectral changes (50 s) of a 33 μM solution of $[\text{Fe}^{\text{III}}(\text{ApT})_2]^+$, and $\alpha\beta\text{-HbO}_2$ (1.5 μM ; 3.0 μM heme). (B) Slow time resolved UV-Vis spectral changes (300 s) of a 27 μM solution of $[\text{Fe}^{\text{III}}(\text{Ap44mT})_2]^+$ and $\alpha\beta\text{-HbO}_2$ (1.5 μM ; 3.0 μM heme). Conditions as those indicated in the Experimental section.

The shoulder remaining at *ca.* 620 nm in Figure 1B is due solely to methHb. These changes may also be appreciated by examining selected time-resolved difference absorption spectra for reactions with $[\text{Fe}^{\text{III}}(3\text{-AP})_2]^+$ measured over 50 s (Supporting Information Figs S1-S3) and 300 s (Figs S4-S6), where an absorbance increase at 620 nm from $[\text{Fe}^{\text{II}}(3\text{-AP})_2]$ is only seen in the early stage of the reaction but over a longer timescale there is no net accumulation of Fe^{II} complex due to Equation 2, where *xx* represents all generic forms of substitution at the N^4 atom.

The $[\text{Fe}^{\text{II}}(\text{ApxxT})_2]$ complexes and $[\text{Fe}^{\text{II}}(3\text{-AP})_2]$ display relatively low $\text{Fe}^{\text{III/II}}$ redox potentials [35] (Table 1) making Equation 2 more rapid for the than for the **HDpT** and **HBpT** series. Intermediate behaviour is found for the **HBpT** series (inset Figure 2B) and the $[\text{Fe}^{\text{II}}(\text{Bp4eT})_2]$ absorption band at 620 nm is persistent even at the end of the run (300 s). For the complex $[\text{Fe}(3\text{-AP})_2]^+$ (Figure 2A), which has a similar redox potential to the $[\text{Fe}^{\text{II}}(\text{ApxxT})_2]$ series, the Fe^{II} complex is only transient and reoxidises during the experiment (Supporting Information Figs S4-S6).

Figure 2. (A) Full spectral changes of a 27 μM solution of $[\text{Fe}^{\text{III}}(3\text{-AP})_2]^+$ and $\alpha\beta$ HbO_2 (1.5 μM ; 3.0 μM heme), total time 50 s (B) Full spectral changes of a 27 μM solution of $[\text{Fe}^{\text{III}}(\text{Bp4eT})_2]^+$, and $\alpha\beta$ HbO_2 (1.5 μM ; 3.0 μM heme), total time 300 s. Conditions as in the Experimental section.

Modelling the time-resolved spectral changes under the conditions indicated in the Experimental section, *i.e.* pseudo first order kinetics, resulted in consistent two consecutive (pseudo) first order steps (Figure 3A). In full agreement with this assumption are the deconvoluted absorption spectra shown in Figure 3B. In the inset to Figure 3B, the calculated spectrum of the intermediate, half-metHbO₂, species corresponds to a composite of the spectra of a 50% HbO₂ and 50% metHb mixture (and the Fe complex which is in excess), which reinforces the assumed independence of the two chromophores. That is, a distinct reactivity of the α - and β -chains is observed [9], which is directly related to the expected cooperative effects in the protein; conformational changes coupled to oxygen release should affect the other chains within the protein. The same phenomenon has already been found for the HDp $\alpha\alpha$ T series [17].

Figure 3. (A) ReactLab Kinetics-calculated concentration-time profiles of HbO₂ (dark red), half-metHbO₂ (green), and metHb (blue). (B) ReactLab Kinetics-calculated spectra for HbO₂ (dark red), half-metHbO₂ (green), and metHb (blue) for the same experiment. Spectral data include the 27 μM flooding contribution of $[\text{Fe}^{\text{III}}(\text{BpeT})_2]^+$ which is constant during the reaction.

The typical dependence of the values determined for k_{obs} versus the (excess) Fe complex concentration is shown in Figure 4, where a clear linear dependence is observed for both the fast (first) and slow (second) oxidation reactions. In all cases, as a general and consistent rule, these plots are consistent with a zero intercept when experimental errors are considered and so all plots were constrained to pass through the origin. This is to be expected as HbO₂ autooxidation is negligible on the timescale of these experiments; the auto-oxidation rate constant is $\sim 10^{-5} \text{ s}^{-1}$ ($t_{1/2} \sim 1 \text{ d}$) for HbO₂ [9,

41]. Furthermore, no significant build-up of any outer-sphere precursor complex ($[\text{HbO}_2]:[\text{Fe}^{\text{III}}\text{L}_2]^+$) is observed, which would otherwise be revealed by curvature of the plots in Figure 4. The presence of dioxygen bubbles (Equation 1) produced baseline instabilities for reactions run over periods of minutes, and a minimum of four replicates were needed to obtain reliable results. Table 1 collects all the second order rate constants (k_f and k_s) slopes determined in this work and those published before [17].

Figure 4. Plots of the observed (pseudo) first order rate constant for HbO_2 oxidation (k_{obs}) as a function of ferric complex concentration for the fast (circles) and slow (squares) steps: (A) $[\text{Fe}^{\text{III}}(\text{BpT})_2]^+$; (B) $[\text{Fe}^{\text{III}}(\text{Ap44mT})_2]^+$; (C) $[\text{Fe}^{\text{III}}(3\text{-AP})_2]^+$.

Many of the redox potentials of Fe complexes in this work have been published before [29, 30, 35, 37], but not all under the same conditions (solvent and supporting electrolyte). In Figure 5 a member from each series of complexes (including $[\text{Fe}(\text{3-AP})_2]^+$) is included where the terminal N-atom (N^4) is unsubstituted. The solvent was MeCN:H₂O (70:30) to ensure solubility of all complexes. In each case, a reversible $\text{Fe}^{\text{III/II}}$ couple is observed at a potential that is determined solely by inductive effects inherent to the carbonyl compound precursor (di-2-pyridyl ketone, 2-benzoylpyridine, 2-acetylpyridine or 3-amino-2-pyridinecarbaldehyde) because the first coordination spheres of all complexes are identical.

Figure 5. Cyclic voltammetry of four representative complexes from this work: (from top to bottom) $[\text{Fe}(\text{DpT})_2]^+$; $[\text{Fe}(\text{BpT})_2]^+$; $[\text{Fe}(\text{ApT})_2]^+$ and $[\text{Fe}(\text{3-AP})_2]^+$. Sweep rate 100 mV s^{-1} , solvent MeCN:H₂O 70:30, supporting electrolyte Et_4NClO_4 (0.1 M) all complexes approximately 1 mM concentration.

As has been observed previously, the $[\text{Fe}(\text{DpT})_2]^+$ complexes exhibit systematically the highest redox potentials of this series due to the electron withdrawing effect of the non-coordinating pyridyl group [29]. The cyclic voltammetry of $[\text{Fe}(\text{3-AP})_2]^+$ has not been reported in mixed aqueous solution, but

only in 100% DMSO [37]. Here the redox potential of $[\text{Fe}(\text{3-AP})_2]^+$ in $\text{MeCN}:\text{H}_2\text{O}$ (70:30) is almost the same as $[\text{Fe}(\text{ApT})_2]^+$, which is among the lowest potential Fe complexes of all the thiosemicarbazones we have investigated. In the context of this work, the Fe^{III} complexes of the **HApT** analogues (and **H3-AP**) are the weakest oxidants from a thermodynamic perspective and their Fe^{II} complexes will be the most reactive towards molecular oxygen as seen for $[\text{Fe}^{\text{II}}(\text{3-AP})_2]$ (Figure 2A) and $[\text{Fe}^{\text{II}}(\text{Ap44mT})_2]$ (Figure 1B) which do not accumulate because the Fe^{II} complex is consumed by dioxygen at a similar rate as it is generated as a product of HbO_2 oxidation.

Table 1. Second order rate constants for the fast and slow steps of the oxidation reaction of the $\alpha\beta\text{-HbO}_2$ by $[\text{Fe}^{\text{III}}(\text{L})_2]^+$ under the conditions indicated in the Experimental section; redox potentials previously published are also included for discussion.

$[\text{Fe}^{\text{III}}(\text{L})_2]^+$	$E'[\text{Fe}^{\text{III/II}}]$ (mV vs NHE)	k_f ($\text{M}^{-1}\text{s}^{-1}$)	k_s ($\text{M}^{-1}\text{s}^{-1}$)
$[\text{Fe}(\text{DpT})_2]^+$	+165	$1.9(6) \times 10^3$	$9.2(4) \times 10^2$
$[\text{Fe}(\text{Dp4mT})_2]^+$	+153	$9.5(4) \times 10^2$	$3.7(1) \times 10^2$
$[\text{Fe}(\text{Dp4eT})_2]^+$	+173	$9.2(3) \times 10^2$	$3.2(1) \times 10^2$
$[\text{Fe}(\text{Dp44mT})_2]^+$	+166	$8.1(4) \times 10^2$	$4.1(1) \times 10^2$
$[\text{Fe}(\text{BpT})_2]^+$	+120	$1.8(5) \times 10^3$	$6.6(1) \times 10^2$
$[\text{Fe}(\text{Bp4mT})_2]^+$	+108	$1.0(2) \times 10^2$	$4.5(1) \times 10^2$
$[\text{Fe}(\text{Bp4eT})_2]^+$	+99	$1.1(3) \times 10^3$	$2.6(2) \times 10^2$
$[\text{Fe}(\text{Bp44mT})_2]^+$	+119	$9.2(6) \times 10^2$	$3.1(2) \times 10^2$
$[\text{Fe}(\text{ApT})_2]^+$	+20	$1.7(1) \times 10^3$	$3.9(1) \times 10^2$
$[\text{Fe}(\text{Ap4mT})_2]^+$	-3	$7.9(3) \times 10^2$	$1.9(1) \times 10^2$
$[\text{Fe}(\text{Ap44mT})_2]^+$	+49	$7.7(4) \times 10^2$	$1.3(1) \times 10^2$
$[\text{Fe}(\text{3-AP})_2]^+$	+47	$1.3(1) \times 10^3$	$3.8(1) \times 10^2$

Discussion

Molecular understanding of HbO₂ oxidation

The high affinity of thiosemicarbazone chelators for iron, as well as the redox-activity of their iron complexes are essential in metHb formation, as already described [4, 29, 30]. For the ferric complexes of the **HDp_{xx}T** analogues, an outer–sphere electron transfer mechanism, between the iron complexes and exposed edge of the heme prosthetic group within the protein, has to be operative for the redox processes studied [17]. In the first instance, an inner-sphere mechanism can be ignored due to the fact that the Fe^{III} complexes bear no labile coordination sites capable of attachment of a bridging ligand. Furthermore, the dimensions of the complexes exceed 10 Å in diameter, which is much too large to enter the heme pocket adjacent to the coordinated dioxygen ligand [29, 30]. For a typical site-specific, outer-sphere electron transfer reaction, a plot of k_{obs} versus iron complex should show curvature[§] and eventually reach a maximum at high (saturating) concentrations of Fe^{III} complex [42, 43], but we observed no evidence of this here. The fact that only a relatively small concentration range of Fe^{III} could be employed due to spectral interference of the thiosemicarbazone complexes (see Experimental section) is the most likely explanation for the apparent linear dependence. Theoretically, at higher Fe^{III} concentrations, curvature of the plots in Figure 4 should be observed [44].

In our initial investigations with complexes from the [Fe(Dp_{xx}T)₂]⁺ family in reaction with HbO₂, we observed no relationship between the Fe^{III/II} redox potential and the rate at which they oxidised the protein [17]. Notwithstanding, the redox potential range examined was very narrow (*ca.* 20 mV), which prompted this study to include members of the [Fe(Bp_{xx}T)₂]⁺ and [Fe(Ap_{xx}T)₂]⁺ series as these complexes show significantly lower redox potentials. The range of redox potentials is now extended to $-3 < E' < +173$ mV *vs* NHE. Clearly, it now emerges that (again) no correlation exists between the Fe^{III/II} redox potentials and the second order rate constants for HbO₂ oxidation (both fast and slow).

[§] the equation $k_{\text{obs}} = K_{\text{OS}} \cdot k^{\text{lim}} [\text{Fe}^{\text{III}}] / (1 + K_{\text{OS}} [\text{Fe}^{\text{III}}])$ applies in this case where K_{OS} is the outer sphere complex formation constant ($\text{HbO}_2 : [\text{FeL}_2]^+$) and k^{lim} is the limiting rate constant at high (saturating) Fe complex concentration. If K_{OS} is very small (or [Fe] very low) then the denominator is unity and the equation becomes $k_{\text{obs}} = K_{\text{OS}} \cdot k^{\text{lim}} [\text{Fe}^{\text{III}}]$ *i.e.* the second order rate constants in Table 1 (k_f and k_s) represent the product $K_{\text{OS}} \cdot k^{\text{lim}}$ in each case.

There are four complexes in Table 1 that stand out as the most potent HbO₂ oxidants; [Fe(DpT)₂]⁺, [Fe(BpT)₂]⁺, [Fe(ApT)₂]⁺ and to a lesser extent [Fe(3-AP)₂]⁺.

Figure 6. Crystal structure of human HbO₂ [45] ($\alpha\beta$ dimer shown, PDB 1HHO). The α -chain is on the left as drawn and the solvent exposed heme propionates are highlighted.

We already noted [17] that the complex [Fe(DpT)₂]⁺ is significantly more reactive than the other [Fe(Dp $\alpha\alpha$ T)₂]⁺ analogues toward HbO₂ oxidation. At the time we speculated the greater reactivity of [Fe(DpT)₂]⁺ was due to its lack of N⁴-terminal substituents enabling closer approach of the Fe complex to the solvent-exposed heme prosthetic groups (Figure 6) and perhaps that the terminal –NH₂ groups might potentially facilitate H-bonding interactions with the heme propionates (Scheme 1). Given that: **(1)** [Fe(DpT)₂]⁺, [Fe(BpT)₂]⁺, [Fe(ApT)₂]⁺ and [Fe(3-AP)₂]⁺ all share this structural feature; **(2)** they all show similarly rapid HbO₂ oxidation kinetics; and **(3)** that their redox potentials are distinctly different, adds support to our initial proposition. It is apparent from Scheme 1 that neither the **R** substituents (which discriminate the **HDpT** (Py), **HBpT** (Ph), **HApT** (Me) series and **H3-AP** (H)) or the **X** substituent at the 3-position of the coordinated pyridine have any bearing on this H-bonding interaction. Thus, the same model may be applied to the four complexes [Fe(DpT)₂]⁺, [Fe(BpT)₂]⁺, [Fe(ApT)₂]⁺ and [Fe(3-AP)₂]⁺.

Conversely, the addition of one or two substituents to the terminal N-atom will weaken (or remove) any possible H-bonding. Considering this, it is notable that thiosemicarbazones with larger substituents have been reported including the ligand, **HDpC**, which bears a methyl and cyclohexyl substituent [4, 32] (Scheme 1). This ligand has shown promising properties as an anti-cancer drug and exhibits very minimal HbO₂ oxidation in intact erythrocytes and *in vivo* in mice [4]. Unfortunately, the Fe^{III} complex $[\text{Fe}(\text{DpC})_2]^+$ (Scheme 1) exhibits low solubility and precipitates HbO₂ when oxidation experiments similar to those reported here are attempted, so we have been unable to directly compare the kinetics of HbO₂ oxidation by $[\text{Fe}(\text{DpC})_2]^+$ with the other compounds in Table 1.

Biological implications

The issues of methemoglobinemia identified through administration of Triapine[®] (**H3-AP**), appear to be based more on its structure than its Fe^{III/II} redox potential. A possible synthetic strategy in terms of lowering this undesirable reactivity would be to introduce substituents on the terminal N⁴ atom of the thiosemicarbazone moiety (as achieved with **HDpC**; Scheme 1). Hence, on the basis of our results, we predict that this would render the compound less toxic. However, Scheme 1 alone does not account completely for the results obtained so far. If H-bonding was necessary for HbO₂ oxidation, then the

complexes $[\text{Fe}(\text{Dp44mT})_2]^+$, $[\text{Fe}(\text{Bp44mT})_2]^+$ and $[\text{Fe}(\text{Ap44mT})_2]^+$ (possessing no NH groups) would be completely unreactive. However, this is not the case, with the rate at which these complexes oxidise HbO_2 being comparable with mono-alkylated analogues such as $[\text{Fe}(\text{Dp4mT})_2]^+$, $[\text{Fe}(\text{Bp4mT})_2]^+$ and $[\text{Fe}(\text{Ap4mT})_2]^+$ (Table 1). Notably, the N-methyl substituents are not nearly as bulky as a cyclohexyl group, so it may merely be steric effects alone that keep $[\text{Fe}(\text{DpC})_2]^+$ far enough away from the heme prosthetic group to attenuate HbO_2 oxidation. In contrast, $[\text{Fe}(\text{Dp44mT})_2]^+$ can still approach the heme edge sufficiently closely to facilitate outer sphere electron transfer.

Conclusions

The kinetics and mechanism of the oxidation of oxyhemoglobin (HbO_2) to methemoglobin (metHb) by biologically active Fe^{III} complexes of the 2-benzoylpyridine thiosemicarbazone series (**HBpT**), 2-acetylpyridine thiosemicarbazone series (**HApT**), and Triapine[®] (**H3-AP**) have been studied. Time-resolved UV-Vis spectral changes obtained are consistent with an intermediate semi-oxidised form of HbO_2 that is further oxidised, in a second slower reaction, to completely oxidised metHb. The two steps have been associated to separate outer-sphere oxidations of the α - and β -chains in the protein as for previously studied Fe -**HDpT** complexes [17]. The $[\text{Fe}^{\text{III}}(\text{BpT})_2]^+$, $[\text{Fe}^{\text{III}}(\text{ApT})_2]^+$ and $[\text{Fe}^{\text{III}}(\text{3-AP})_2]^+$ complexes show a two-fold enhanced oxidation rate of the $\alpha\beta$ - HbO_2 protein compared with the rest of the complexes from their respective series that bear substituents on the terminal N-atom of the thiosemicarbazone group. This difference has been associated with the combination of favourable steric and hydrogen-bonding effects that enable a closer and more efficient approach between the iron thiosemicarbazone complex and the heme prosthetic group.

Table of Abbreviations

HbO₂: human oxyhemoglobin

metHb: human methemoglobin

HDpT: di-2-pyridylketone thiosemicarbazone (protonated form)

HBpT: 2-benzoylpyridine thiosemicarbazone

HApT: 2-acetylpyridine thiosemicarbazone

H3-AP 3-amino-2-pyridinecarboxaldehyde thiosemicarbazone

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Chart 1

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Table 1. Second order rate constants for the fast and slow steps of the oxidation reaction of the $\alpha\beta$ -HbO₂ by [Fe^{III}(L)₂]⁺ under the conditions indicated in the Experimental section; redox potentials previously published are also included for discussion.

[Fe ^{III} (L) ₂] ⁺	$E'_{[\text{Fe}^{\text{III}}]}$ (mV vs NHE)	k_f (M ⁻¹ s ⁻¹)	k_s (M ⁻¹ s ⁻¹)
[Fe(DpT) ₂] ⁺	+165	$1.9(6) \times 10^3$	$9.2(4) \times 10^2$
[Fe(Dp4mT) ₂] ⁺	+153	$9.5(4) \times 10^2$	$3.7(1) \times 10^2$
[Fe(Dp4eT) ₂] ⁺	+173	$9.2(3) \times 10^2$	$3.2(1) \times 10^2$
[Fe(Dp44mT) ₂] ⁺	+166	$8.1(4) \times 10^2$	$4.1(1) \times 10^2$
[Fe(BpT) ₂] ⁺	+120	$1.8(5) \times 10^3$	$6.6(1) \times 10^2$
[Fe(Bp4mT) ₂] ⁺	+108	$1.0(2) \times 10^2$	$4.5(1) \times 10^2$
[Fe(Bp4eT) ₂] ⁺	+99	$1.1(3) \times 10^3$	$2.6(2) \times 10^2$
[Fe(Bp44mT) ₂] ⁺	+119	$9.2(6) \times 10^2$	$3.1(2) \times 10^2$
[Fe(ApT) ₂] ⁺	+20	$1.7(1) \times 10^3$	$3.9(1) \times 10^2$
[Fe(Ap4mT) ₂] ⁺	-3	$7.9(3) \times 10^2$	$1.9(1) \times 10^2$
[Fe(Ap44mT) ₂] ⁺	+49	$7.7(4) \times 10^2$	$1.3(1) \times 10^2$
[Fe(3-AP) ₂] ⁺	+47	$1.3(1) \times 10^3$	$3.8(1) \times 10^2$

Highlights

- Heterocyclic thiosemicarbazone Fe chelators are promising anti-cancer agents.
- Some chelators show severe side effects such as methemoglobinemia.
- Reactivity of their Fe(III) complexes toward hemoglobin oxidation is the cause.
- Steric effects and not the Fe(III/II) redox potential dominate reactivity.
- Design elements are identified for future drug design in this class of anticancer compound.