Modulation of the oxygen affinity of cobalt-porphyrin by globin

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Abstract We have combined two extreme effects which influence the oxygen affinity to obtain a cobalt-based oxygen carrier with an affinity similar to that of human adult hemoglobin (HbA). The goal was to obtain an oxygen transporter with a lower oxidation rate. Exchange of the heme group (Fe-protoporphyrin IX) in Hb with a cobalt-porphyrin leads to a reduction in oxygen affinity by over a factor of 10, an oxygen affinity too low for use as a blood substitute. At the other extreme, certain globin sequences are known to provide a very high oxygen affinity; for example, Hb *Ascaris* displays an oxygen affinity 1000 times higher than HbA. We demonstrate here that these opposing effects can be additive, yielding an oxygen affinity similar to that of HbA, but with oxygen binding to a cobalt atom. We have tested the effect of substitution of cobalt-porphyrin for heme in normal HbA, sperm whale (SW) Mb (Mb), and high affinity globins for leghemoglobin, two trematode Hbs: *Paramphistomum epiclitum* (*Pe*) and *Gastrothylax crumenifer* (*Gc*). As for HbA or SW Mb, the transition from heme to cobalt-porphyrin in the trematode Hbs leads to a large decrease in the oxygen affinity, with oxygen partial pressures for half saturation (P_{50}) of 5 and 25 mm Hg at 37°C for cobalt-*Pe* and cobalt-*Gc*, respectively. A critical parameter for Hb-based blood substitutes is the autoxidation rate; while both metals oxidize to an inactive state, we observed a decrease in the oxidation rate of over an order of magnitude for cobalt versus iron, for similar oxygen affinities. The time constants for autoxidation at 37°C were 250 and 100 h for *Pe* and *Gc*, respectively.

Key words: Hemoglobin; Cobalt prophyrin; Blood substitute; Oxidation rates

1. Introduction

Acellular blood substitutes have entered the various phases of clinical trials; while early studies showed that unmodified hemoglobin (Hb) solutions are nephrotoxic, major improvements in the properties and lifetime were obtained by use of Hb that was crosslinked to stabilize the tetrameric form [1,2]. Certain secondary effects such as vasoconstriction have been reported, probably due to the reaction between Hb and NO, the messenger in the endothelial cells for vasodilatation. Other oxidation reactions or bacterial growth may also be enhanced due to the iron and hemin available from the degrading Hb solution [1,2]. While much progress has been made in regulating the oxygen affinity and stabilizing the tetrameric form, the problems of oxidation of the Hb and related reactions due to the degradation of the Hb remain [3].

The purpose of the present study was to determine whether an oxygen binding protein could be designed with cobalt rather than the usual iron atom of Hb. Hb and Mb with substitution of Fe for Co have been studied for several years. These proteins and Co/Fe-Hb hybrids have been useful for understanding the internal mechanisms of ligand binding to Hb tetramers. Essentially, the replacement of Fe with Co decreases the oxygen affinity (Fig. 1) by a factor of 50 for Mb, and a factor of 12 for Hb [4]. Under physiological conditions, the cobalt-HbA would be partially liganded at the lungs, and would not deliver sufficient oxygen.

Another mechanism of interest for protein engineering involves the very high oxygen affinity observed for hemoproteins that function in a low oxygen environment, such as Hb *Ascaris* or the Hb from trematodes. The discovery that the substitution in position B10 of sperm whale (SW) Mb leads to a higher oxygen affinity (Fig. 1) and lower oxidation rate revealed the site responsible for the high affinity species [5]. Subsequent studies on Hb *Ascaris* showed that the oxygen affinity could be decreased by modifying this same position [6]. The residue B10 is involved in the molecular mechanism via an additional stabilizing H-bond with the oxygen molecule. The overall oxygen affinity of hemoproteins is controlled by the number of bonds with the oxygen molecule: in addition to the bond with the iron atom, there may be as many as two hydrogen bonds between the oxygen molecule and protein residues. Tertiary changes can then be made to fine tune the oxygen affinity. In the case of tetrameric Hb, changes in the allosteric equilibrium will also modify the overall affinity. The large change in oxygen affinity (of a factor of 100) between the two allosteric conformations raises the question as to whether the H-bond with the distal residue is still intact for Hb in the low affinity deoxy (T-state) conformation.

The changes due to different effectors and/or mutations may have additive effects on the oxygen affinity. By combining several effects, one can vary the oxygen affinity by nearly a factor of a million. The aim of the present study was to see whether a very high oxygen affinity globin could be combined with the low intrinsic oxygen affinity cobalt-porphyrin to obtain a cobalt-based oxygen carrier with a moderate oxygen affinity.

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2. Materials and methods

HbA was isolated from the hemolysate of blood from non-smoking healthy donors. Soybean leghemoglobin (Lb) was prepared as described previously [7].

Trematode Hbs were recovered from a slaughter house in Aligarth, India; these monomeric Hbs were purified as described previously [8]. For the present studies, two species were tested: *Paramphistomum epiclitum* (*Pe*) and *Gastrothylax crumenifer* (*Gc*). For Hb *Gc*, the two main electrophoretic components were tested.

Cobalt-porphyrin from Porphyrin Products (Logan, UT, USA) was dissolved in 0.1 M NaOH. A stock solution of about 200 μM Co-porphyrin was then prepared in 50% aqueous/pyridine solution.

2.1. Cobalt-porphyrin proteins

The classical method of Yonetani [4] involves protein precipitation to obtain the apo-globin (without porphyrin); a holo-protein is then formed by reconstitution with the new porphyrin. To avoid the problems involved with protein precipitation and refolding, we devised an alternate method based on porphyrin exchange. At the pH extremes, the protein unfolds and separates from the heme. By working near the limit of unfolding, the exchange can be accomplished without significant unfolding or denaturation. Since the Co-porphyrin is soluble at alkaline conditions, we introduced a 10-fold excess of Co-porphyrin at pH 10.5, under anaerobic conditions with a slight excess of Na-dithionite to reduce the metal atoms. The samples were incubated at the elevated pH for 10–20 min to allow the porphyrin exchange, brought to about pH 7.5 with phosphate buffer (at pH 6), and finally passed through a Sephadex G-25 column (at pH 7) to remove excess porphyrins and dithionite. The advantage of this exchange method is the speed and the fact that small quantities can be prepared as needed. Absorption spectra (SLM-Aminco) under various gas partial pressures (oxygen, argon, CO) were measured to control the overall quality. Based on the absorption spectra with and without oxygen, the final protein was about 90% Co-porphyrin.

2.2. Oxygen affinity

The native hemoproteins *Pe* and *Gc* have an oxygen affinity too high for determination using the usual equilibrium methods. These forms were studied by the ligand binding kinetics. Oxygen on-rates were determined by flash photolysis. The dissociation rates were measured by stopped flow by mixing the oxy species with a solution of Na-dithionite. For moderate oxygen affinity species, measuring full absorption spectra at a few oxygen partial pressure values using a tonometer allowed a determination of the equilibrium coefficient. When possible ($P_{50} > 1$ mm Hg), the oxygen equilibrium curves of the modified forms were measured on a Hemox analyzer.

2.3. Oxidation kinetics

For the hemoproteins, the oxidation process can be followed by the change in the absorption versus time, since the oxidized and oxy forms have distinct spectra. This simple method cannot be applied to the cobalt forms, since the spectra for the oxygen bound and the oxidized (Co^{3+}) form are quite similar. The difference spectrum between forms equilibrated under oxygen and nitrogen (or argon) is the evidence for an active oxygen binding species. The absorption spectra were measured under oxygen, then argon to obtain the difference spectrum (oxy versus deoxy). The amplitude of this absorption difference versus time was used to determine the oxidation kinetics for the cobalt-porphyrin proteins.

3. Results and discussion

The native trematode Hbs showed very high oxygen affinities. The high affinity is due to a combination of an elevated oxygen on-rate (as for Lb) with a low dissociation rate. After exchange with Co-porphyrin, the proteins showed the same spectral characteristics as for similar studies with HbA or SW Mb (Fig. 2), notably the transition from iron to cobalt induces a moderate red shift in the oxy Soret band (from 415 to 425 nm) and a large blue shift of the deoxy Soret band (from 432 to 405 nm).

The native trematode Hbs are resistant to oxidation, as previously reported for another high oxygen affinity species, Lb [9]. In general, there is a correlation between the oxygen affinity and the rate of autoxidation [10,11]. As for the high oxygen affinity Mb mutant (L29F), the trematode Hbs require over 100 h for autoxidation at 37°C (Fig. 3).

As for previous studies with Hb and Mb, the substitution of Co for Fe in *Pe* globin leads to a large decrease in the oxygen affinity. Thus these effects are additive and a variety of affinities can be obtained by combining various globins, metals, effectors or crosslinking agents. The ratio of oxygen affinity for Fe versus Co was higher for the trematodes than for HbA; a similar result was obtained for Lb [12]. The cobalt atom may also perturb the H-bonds with the oxygen molecule to induce such a large ratio of affinities.

Cobalt is naturally present as vitamin B12 (cobalamin). In this case, the cobalt atom is at the center of a corrin ring, similar to the porphyrin ring. Thus cobalt itself is not considered toxic, but the larger amounts required for a blood substitute need to be considered. The gradual release of cobalt would provide a different set of conditions from that of iron; one should consider the effects on the erythropoietin gene, shown to be sensitive to $CoCl_2$ at concentrations in the order of 100 μM [13].

For use as a blood substitute, several factors must be considered. The oxygen affinity is obviously of primary importance, but the size, oxidation rate and interaction with other molecules are also important.

3.1. Oxygen affinity

The physiological affinity of Hb within red blood cells is about 30 mm Hg. Mimicking the exact properties of HbA is not necessarily the best strategy. In fact, more oxygen can be delivered with an even lower affinity [14]. However, this tends to accelerate the oxidation process, so values between 20 and 30 mm Hg may be the best compromise considering both parameters. The oxygen affinity is perhaps the parameter that has seen the most progress (Fig. 1); various mutations and crosslinks have provided a wide range of oxygen affinities [3].

Effect of pH. The present results with iron- or cobalt-substituted Hb *Pe* showed little change in oxygen binding parameters from pH 6 to 7; an increase in oxygen affinity of a factor of 1.8 at pH 8.5 relative to pH 7 was observed for cobalt-Hb *Pe*. This contrasts with results on cobalt-Lb which showed a 4-fold decrease in oxygen affinity (entirely due to a change in oxygen off-rate) upon increasing the pH, with a p*K* for the transition of 5.7 [12]. The result for Lb suggested a correlation with the protonation of the distal histidine residue at acid pH; this would be consistent with the present results, as Hb *Pe* has a tyrosine at both critical positions thought to interact with the bound oxygen.

3.2. Size of the oxygen transporter

The size of the transporter is also critical, as dimers of 32 kDa are rapidly eliminated from the bloodstream. Initial clinical studies with unmodified HbA showed a clearance in less than 1 h, leading to nephrotoxicity [1]. Clinical studies with crosslinked Hb have shown an improved lifetime. Thus a Hb tetramer appears to be the minimum size for an acellular transporter. There are several types of crosslinked or polymerized Hb which may overcome this problem.

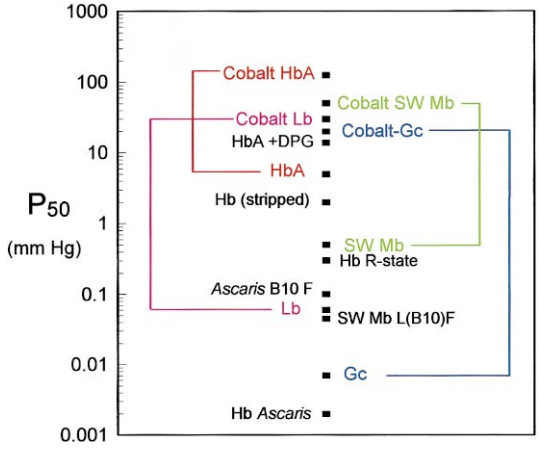

Fig. 1. Oxygen affinity of various natural and modified proteins, as indicated by P_{50}, the oxygen partial pressure necessary for 50% saturation. Values are for experiments at 25°C; the very high affinities (low P_{50}) were determined from the ligand binding rates. Data are for Hb *Ascaris* [6], cobalt-Hb and Mb [4], cobalt-Lb [12] and for the trematode species *Gc* (present study). The lines show the difference (for the same globin) for the transition from iron- to cobalt-based oxygen carrier.

The trematode globins used in this study are similar in size to Mb. The use of monomeric proteins of this small size in vivo is not feasible. A crosslinked or polymerized sample would be necessary to increase the overall size of the transporter. An alternative would be to introduce the necessary mutations in the α and β chains of human globin, as accomplished for SW Mb [11]. This would provide the minimum size, and hopefully maintain the Hb cooperativity as well. While cooperative ligand binding is not essential, oxygen delivery is more efficient, and the high affinity allosteric state helps to maintain a lower autoxidation rate in Hb. Use of a polymer of identical subunits would have the advantage of avoiding the reaction with haptoglobin, which binds Hb dimers irreversibly in the blood plasma.

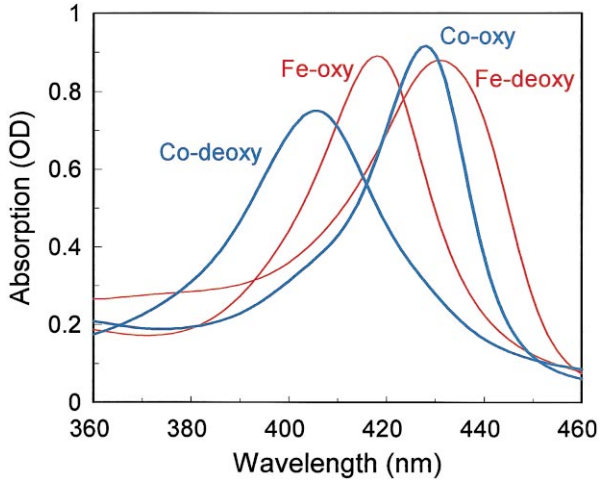

Fig. 2. Absorption spectra of Fe- and Co-porphyrin-substituted *Gc* trematode, for solutions equilibrated under oxygen or nitrogen (for the deoxy form). Measurement of the difference spectrum versus time was used to determine the oxidation rate.

3.3. Oxidation rate

Another important parameter for a blood substitute is the autoxidation rate, since the oxidized form no longer binds oxygen. Even if the Hb remains in circulation, the oxidation rate will limit its useful lifetime. Without the met-Hb reductase enzymatic system that is present in the red blood cells, the autoxidation may be irreversible. Not only will the number of oxygen carriers decrease with time, but for Hb tetramers, a single oxidized subunit can perturb the oxygen affinity for the neighboring chains. Overall, the loss in oxygen delivery of Hb is approximately twice the amount oxidized; that is, for 10% oxidized subunits, a 20% loss in oxygen delivery is estimated [14]. This could be compensated for by a very low affinity Hb (beyond the peak value of P_{50} for maximum oxygen delivery) so that the decrease in P_{50} due to partial oxidation would increase the delivery; however, low affinity Hbs have shown an increased propensity to oxidation. This is in part due to a fundamental correlation between the affinity and rate of autoxidation as shown for Mb mutants [10], and also because the partially liganded Hb tetramers oxidize more rapidly, probably due to the contribution of the low affinity T-state.

The high oxygen affinity species, and the Co-Hb are both known to be stable. It was hoped that the Co proteins with moderate oxygen affinity would show some improvement in the oxidation problem. As for iron-based proteins, these Co-porphyrin globins showed a decrease in oxygen binding signal (difference spectrum between samples equilibrated under oxygen and nitrogen) versus time. However, for a similar value of the oxygen affinity, the oxidation rates were over an order of magnitude lower (Fig. 3). While HbA shows about 12% oxidation (loss of nearly 20% oxygen delivery) within 8 h, the Co-based transporter would show a loss of less than 4%. The oxidation during storage also plays a role in determining the oxidation level just before use.

While genetic engineering and crosslinking techniques have shown several solutions to regulate the oxygen affinity and stabilize the tetramers, control of the oxidation rate of these

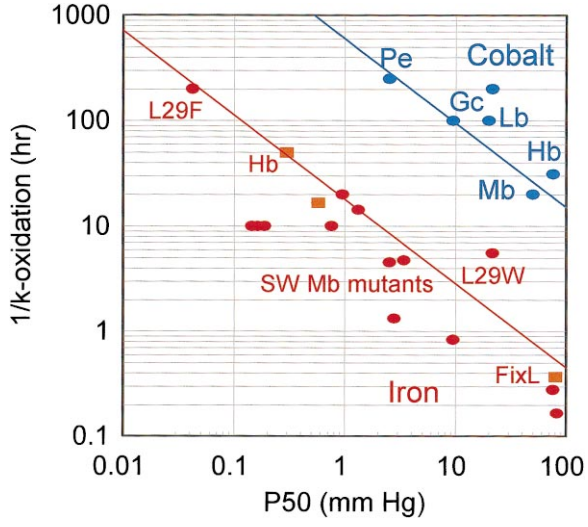

Fig. 3. Oxidation rate (at 37°C) versus P_{50} (partial pressure of oxygen for half saturation, determined at 25°C) for Fe- and Co-based oxygen carriers. Data for Mb mutants (iron) from Olson et al. [11]; native proteins Mb, Fix-L and R-state Hb are also shown (squares). Values below the (arbitrary) lines of slope 1 indicate a faster autoxidation.

proteins remains a challenge. If tolerated by the organism, the Co-porphyrin could provide a major improvement in this parameter.

3.4. Binding of carbon monoxide (smoker's globin)

One of the advantages of these Co-substituted proteins is that they do not bind CO as ligand. As for Hb tetramers that are partially oxidized, a small percentage of CO bound subunits can perturb the neighboring chains. For example, if randomly distributed, 2% of sites with CO bound could correspond to nearly 8% of the tetramers with at least one subunit affected; these tetramers would not make the full transition to the deoxy state and thus be less efficient in oxygen delivery.

Cobalt-porphyrins bind NO. As for Hb, use as a blood substitute may induce a vasoconstriction due to depleting the NO supply which acts as a signal in the endothelial cells. A second aspect is the induced oxidation of the iron or cobalt by NO in the presence of oxygen [11].

3.5. Oxidative stress

Hemin and free iron are known to participate in various oxidation–reduction reactions. When used as a blood substitute, the degradation of the Hb releases hemin and iron which may lead to undesirable reactions with membranes or other proteins.

The use of Hb solutions may antagonize situations involving exposure to bacteria. The oxidation and subsequent degradation of the Hb may supply iron that can accelerate bacterial growth [15]. The use of cobalt in place of the iron atom, or a mixture of both types of carrier, could also have advantages concerning these secondary reactions.

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