HEMOGLOBIN-BASED BLOOD SUBSTITUTES: WHICH HEMOGLOBIN TO USE?

FLORINA-VIOLETA DEAC¹, ANA MARIA BOLFA¹, CRISTIAN MAGDAS², BOGDAN SEVASTRE², SILVIA TURC², RADU SILAGHI-DUMITRESCU¹*

¹Department of Chemistry and Chemical Engineering, “Babeş-Bolyai” University, 11 Arany János str., Cluj-Napoca RO-400028, Romania
²University of Agricultural Sciences and Veterinary Medicine, 3–5 Mănăştur Str., Cluj-Napoca 400372, Romania

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Bovine and human hemoglobin (Hb) are known to catalyze peroxide reduction by ascorbate, with high affinity for the latter substrate. It was proposed that such reactivity would be important in hemoglobin-based artificial oxygen carriers, and would be involved in their known toxic side-reactions. The hemoglobin-based blood substitutes proposed to date are based on either human hemoglobin (in limited supply) or bovine hemoglobin (readily available in large amounts). Here, the ascorbate peroxidase reactivity of several hemoglobins is examined, in an attempt to identify cases where the turnover rates or affinities for substrates would be different from those of human or bovine hemoglobin, thereby possibly providing an additional advantage for preparation of a blood substitute.

Key words: hemoglobin, ascorbate, oxidative stress, heme proteins, peroxidase, antioxidants, blood.

INTRODUCTION

Globins are known to react with peroxides, generating high-valent states of biological relevance; we and others have recently reported that in vitro such reactions may be coupled to a further reductive step (e.g., with ascorbate), thereby establishing a true catalytic cycle wherein ascorbate efficiently protects globins from peroxide – induced free radical damage (1–3). Human and bovine hemoglobin as well as horse heart myoglobin were shown to act as ascorbate peroxidases, with well-measurable Michaelis-Menten parameters, which included $K_m$ values close to physiological ranges (2, 3). Furthermore, consistent with this low $K_m$, NMR spectra demonstrated an adduct between ascorbate and hemoglobin (3). We have previously hypothesized (3), based on extensive data and proposals from other groups as well (4), that such

⁵Corresponding author (E-mail: rsilaghi@chem.ubbcluj.ro; Phone: +40-264-593833; Fax: +40-264-590818)

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reactivity against ascorbate and peroxide would be important in acellular hemoglobin-based blood substitutes, to the extent to which it would be involved in side-reactions which have so far led to the failure of these artificial blood substitutes in several clinical tests (4). Most hemoglobin-based blood substitutes employ human hemoglobin (for compatibility reasons) or bovine hemoglobin (due to its abundance as well as due to its non-dependence of the physiological effector, bisphosphoglycerate (4–7). Here, we investigate several hemoglobins from the point of view of their peroxide/ascorbate-related reactivity, and seek to identify cases where the affinity for peroxide or ascorbate, or indeed the turnover rates, are particularly different from those of previously examined hemoglobins and hence might provide an additional advantage when using that particular hemoglobin as raw material for blood substitute preparation.

MATERIALS AND METHODS

Hemoglobins from rat, dog, sheep, cow, and horse were purified as previously described (3) following a general protocol of Antonini and Brunori (8). Thus, the blood, freshly drawn on citrate, was centrifuged 15 minutes at 5000 rpm to separate the red blood cells, which were then washed three times with 5 mM phosphate pH 7.4 + 150 mM NaCl. Hemoglobin concentrations in text are given per heme rather than per tetramer. The met forms of the hemoglobins were prepared by ferricyanide treatment as previously described (9–11).

Ascorbate peroxidase activity was measured as previously described (3), monitoring the time course of absorbance at 290 nm (2, 12). At this wavelength the reduced form of ascorbate shows absorbance, whereas the oxidized form (dehydro-ascorbate) does not; as a consequence, the absorbance decreases linearly in time, from the moment the three key components (ascorbate, peroxide, and catalyst-hemoglobin) are mixed in the UV-vis cuvette. Figures 1 and 2 show measurements of reaction rates as a function of substrate concentrations; the units are expressed as “µM ascorbate consumed per minute”, although they could, in principle, also be expressed as “µM dehydro-ascorbate formed per minute”. Data were fit to simple Michaelis-Menten equations, using a least-squares procedure and the Solver module within the Microsoft Excel software package.

UV-vis spectra were recorded on Agilent 8453 (Agilent, Inc.) and Cary 50 (Varian, Inc) instruments.

RESULTS AND DISCUSSION

Figures 1 and 2 show turnover data for peroxide reduction by ascorbate catalyzed by four hemoglobins (sheep, dog, horse and rat). These plots are in general agreement
Fig. 1. – Dependence of rat (A), horse (B), dog (C), and sheep (D) hemoglobin-catalyzed ascorbate consumption rate on peroxide concentration. At pH 7.4, 50 mM phosphate, 12 µM Hb, room temperature; top panel – 350 µM ascorbate; bottom panel – 800 µM peroxide. The curves show fits of experimental data to Michaelis-Menten kinetics as detailed in Materials and Methods.
Fig. 2. – Dependence of rat (A), horse (B), dog (C), and sheep (D) hemoglobin-catalyzed ascorbate consumption rate on ascorbate concentration. At pH 7.4, 50 mM phosphate, 12 µM Hb, room temperature; top panel – 350 µM ascorbate; bottom panel – 800 µM peroxide. The curves show fits of experimental data to Michaelis-Menten kinetics as detailed in Materials and Methods.
with previously reported data on human and bovine hemoglobin (2, 3) and are therefore expected to involve a peroxidase-type reaction where ascorbate and peroxide bind sequentially to the protein. Table 1 summarizes the $K_m$ and $k_{cat}$ parameters for ascorbate and for peroxide; similarly to the case of bovine Hb, a low $K_m$ for ascorbate is found, at 20–30 µM (2, 3). Also in agreement with data known for human and bovine Hb, the $K_m$ values for peroxide are between 400 and 4000 µM, in line with parameters of similar magnitude reported for bona fide peroxidases (2, 3, 13). The actual value of the ascorbate $K_m$ is expected to be somewhat higher than the ~20 µM measured here under non-saturating peroxide; nevertheless, since measurements for all hemoglobins were done under similar conditions, comparison of their kinetic parameters appears warranted.

Although the kinetic parameters shown in Table 1 are generally similar among the hemoglobins examined here, one can identify cases where some difference exists at least in term of one of the parameters. Thus, bovine and rat Hb appear to have the smallest $K_m$ for peroxide, ~5 times smaller than that of the dog Hb. One may therefore expect that the bovine protein, by virtue of its higher affinity for peroxide, be a less useful material for blood substitutes compared to dog hemoglobin. In terms of $k_{cat}$ values, ovine and rat hemoglobin appear the least reactive towards peroxide. These data may be taken to suggest that dog and sheep hemoglobins would have an advantage over bovine hemoglobin insofar, as they would display less pro-oxidant reactivity. On the other hand, the differences between ascorbate $K_m$ parameters are negligible.

One may attempt to identify structural factors controlling the differences in peroxidase reactivity among the hemoglobins examined here. Previous studies have identified aromatic amino acids controlling the reaction between oxidized hemoglobin and ascorbate (14). Among these residues, tyrosine 42 appeared to be the most important, and was identified as the point where electrons from exogenous reducing agents (such as ascorbate) enter the protein and are directed towards the heme (14). Figure 3 shows the structure of the human α hemoglobin subunit, illustrating that among the tyrosines present in this polypeptide, Tyr42 is

<table>
<thead>
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<th></th>
<th>Human*</th>
<th>cow</th>
<th>horse</th>
<th>dog</th>
<th>sheep</th>
<th>rat</th>
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<tr>
<td>ascorbate $K_m$</td>
<td>191 µM</td>
<td>21 µM</td>
<td>21.3 µM</td>
<td>28 µM</td>
<td>34.6 µM</td>
<td>29 µM</td>
<td>154 µM</td>
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<tr>
<td>peroxide $K_m$</td>
<td>246 µM</td>
<td>734 µM</td>
<td>3277 µM</td>
<td>3950 µM</td>
<td>1702 µM</td>
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<td>390 µM</td>
</tr>
<tr>
<td>$k_{cat}$</td>
<td>15 min$^{-1}$</td>
<td>136 min$^{-1}$</td>
<td>102 min$^{-1}$</td>
<td>88 min$^{-1}$</td>
<td>35 min$^{-1}$</td>
<td>34.4 min$^{-1}$</td>
<td>117 min$^{-1}$</td>
</tr>
</tbody>
</table>

*data from Ref. 2
by far the closest to the heme. As detailed in the figure legend, the distance between the heme and Tyr 42 is the longest in dog Hb, which, interestingly, was also the protein exhibiting the smallest reactivity of those examined by us experimentally, as discussed above.

Fig. 3. – Structure of the hemoglobin α monomer, illustrating the relative positions of the three tyrosine residues believed to be important for electron transfer during the ascorbate peroxidase turnover of hemoglobins. The Tyr42-heme distances are 5.15, 5.5, 5.6 and 5.45 in bovine, horse, dog and ovine hemoglobins, respectively – based on the respective crystal structures, pdb codes 2QSP (bovine), 2YLW (horse), 2QLS (dog), and 2QUO (ovine).

CONCLUSIONS

To conclude, the ascorbate peroxidase reactivity was investigated in several hemoglobins. Judging in terms of affinity towards peroxide and $k_{cat}$ magnitudes, dog and sheep hemoglobin appear to be the least reactive of those examined here, while bovine hemoglobin would be the most reactive. It remains to be established to what extent these differences in reactivity can affect the outcome of a
transfusion experiment performed with blood substitutes prepared by similar recipes using the two different hemoglobins.

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REFERENCES


