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Research Article

Catalase-like activity of bovine met-hemoglobin: Interaction with the pseudo-catalytic peroxidation of anthracene traces in aqueous medium

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Hemoglobin is a member of the hemoprotein superfamily whose main role is to transport O_2 in vertebrate organisms. It has two known promiscuous enzymatic activities, peroxidase and oxygenase. Here we show for the first time that bovine hemoglobin also presents a catalase-like activity characterized by a V_{max} of 344 μM/min, a K_M of 24 mM and a k_{cat} equal to 115/min. For high anthracene and hemoglobin concentrations and low hydrogen peroxide concentrations, this activity inhibits the expected oxidation of anthracene, which occurs through a peroxidase-like mechanism. Anthracene belongs to the polycyclic aromatic hydrocarbon (PAH) family whose members are carcinogenic and persistent pollutants found in industrial waste waters. Our results show that anthracene oxidation by hemoglobin and hydrogen peroxide follows a typical bi-bi ping-pong mechanism with a V_{max} equal to 0.250 μM/min, $K_{M(H2O2)}$ of 80 μM, $K_{M(ANT)}$ of 1.1 μM and k_{cat} of 0.17/min. The oxidation of anthracene is shown to be pseudo-catalytic because an excess of hemoglobin and hydrogen peroxide is required to make PAH completely disappear. Thus, bovine hemoglobin presents, in different degrees, all the catalytic activities of the hemoprotein group, which makes it a very interesting protein for biotechnological processes and one with which structure-activity relationships can be studied.

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1 Introduction

Hemoglobin (Hb) is a well-known protein. It is a heterotetramer that consists of two alpha and two beta subunits (α_2/β_2), each one containing the prosthetic group protoporphyirin IX [1]. This peculiar structure allows Hb to bind, transport and release oxygen in all vertebrate organisms. Depending on the iron atom oxidation state, different types of Hb can be found, notably oxy-, deoxy- and met-hemoglobin [2, 3]. The met-hemoglobin (met-Hb), produced *in vivo* through an autoxidation process, is

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Abbreviations: ANT, anthracene; **CYP**, cytochrome P450; **Hb**, hemoglobin; **HRP**, horseradish peroxidase; **PAH**, polycyclic aromatic hydrocarbons; **UFLC**, ultrafast liquid chromatography

characterized by a ferric (Fe^{III}) state of the hemin and is unable to bind oxygen.

Hb belongs to the hemoprotein superfamily, which includes transport proteins such as globin, responsible for O_2 transport and storage, cytochrome, which is responsible for electron transport, and nitrophorine, a protein responsible for nitric oxide transport [4], and also three catalytic proteins (enzyme) classes named heme-thiolates, peroxidases and catalases.

- Heme-thiolate (EC 1.14) is the recommended collective name for the class of proteins that includes the cytochrome P450 enzymes (CYP). The most common reaction catalyzed by these enzymes is monooxygenation, defined as the insertion of one atom of oxygen into a substrate from molecular oxygen, with the concomitant release of water [5–8] (Fig. 1).
- Peroxidases (EC 1.11.1) use hydrogen peroxide (H_2O_2) as an electron acceptor to catalyze oxidative reactions [9–13] (Fig. 1).
- Catalases, which belong to a sub group of peroxidase (EC 1.11.1.6), catalyze the disproportionation reaction of H_2O_2. In this case, H_2O_2 acts both as an electron acceptor and an electron donor at the same time. The reaction yields molecular oxygen (O_2) and water [14] (Fig. 1).

The hemoprotein family is a very interesting group of proteins for structure-activity relationship studies because the active center, iron porphyrin, is the same for all of the members, and the different activities arise from structural and conformational properties of the protein [15].

Most of the hemoprotein family members exhibit multiple types of activity. In 1994, Sun *et al.* [16] pointed out that chloroperoxidase from *Caldariomyces fumago* (EC 1.11.1.10) had a catalase-like activity. In 2001, Hernandez-Ruiz *et al.* [17] demonstrated the same phenomenon for the horseradish peroxidase (HRP; EC 1.11.1.7). On the other hand, catalases also show peroxidase activities [18]. In 1979, a new group of hemoprotein was discovered and named catalase-peroxidase (KatGs) [19]. KatGs are found in prokaryotes and fungi. They display a predominant catalase activity and a weak peroxidase activity with a broad specificity [20]. Nevertheless, on the basis of their primary sequence KatGs have been recognized to be part of the class I peroxidase super family [21]. All these elements tend to show that different hemoprotein activities may coexist, and are highly dependent on their 3-D structure and on experimental conditions.

Although the principal function of Hb is to transport oxygen, it has been shown that this protein has other functions [22], such as pseudo-enzymatic activities [23–25]. Oxyhemoglobin, in the presence of CYP reductase and the nicotinamide adenine dinucleotide phosphate (NADPH) cofactor, hydroxylates aromatic substrates such as aniline through a CYP-like oxygenase mechanism [26–28]. In the presence of H_2O_2, met-Hb, can catalyze oxygenation of various substrates like 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) and polycyclic aromatic hydrocarbons (PAH) through a peroxidase-like mechanism [29–31]. All these different activities of Hb are assumed to be the result of the heme ability to activate and react with oxygen species.

In the present paper, we report for the first time that Hb, in addition of its oxygenase- and peroxidase-like activities, also presents a catalase-like activity. This oxygen-producing activity has been pointed out during the study of anthracene (ANT) oxidation through the peroxidase pathway of Hb. Anthracene belongs to the PAH family, which are

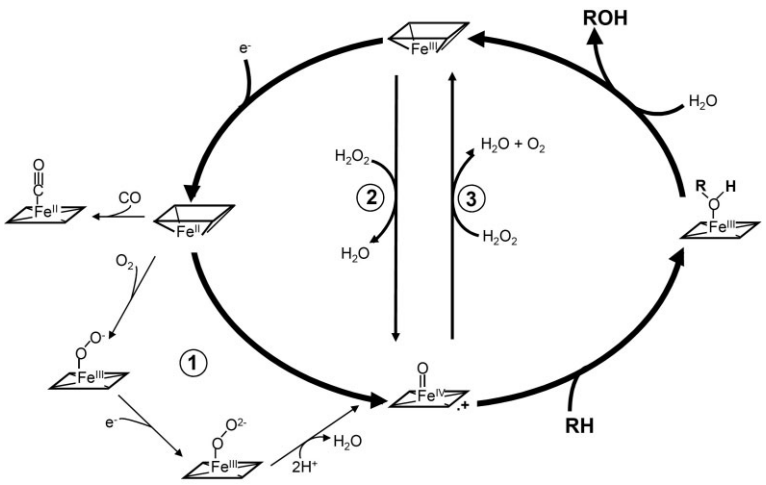

Figure 1. Catalytic cycles of hemoproteins (squares represent the heme group). (1) CYP monooxygenation cycle: the insertion of one atom of oxygen into a substrate RH from molecular oxygen with the concomitant release of water through a complex pathway needing electron and proton transfer before obtaining the active compound $Fe^{IV}=O^{\cdot+}$, which reacts with RH to gives ROH (this pathway can be quenched by the presence of carbon monoxide). (2) In the peroxidase pathway, H_2O_2 oxidizes directly Fe^{III} into the active compound $Fe^{IV}=O^{\cdot+}$ which then reacts with RH. (3) The catalase pathway: the active compound $Fe^{IV}=O^{\cdot+}$, formed by the action of a first H_2O_2 molecule, can react with a second H_2O_2 molecule and lead to the release of O_2 and H_2O.

carcinogenic and mutagenic pollutants found as traces (ng to µg/L) in waste waters from petroleum refineries [32]. Bovine met-Hb is a cheap material that comes from the food industry and is a very promising candidate for bioremediation of PAH in water. Here we present a detailed study of ANT oxidation mechanism by met-Hb and H_2O_2 revealing that in aqueous medium ANT oxidation is a "pseudo-catalytic" reaction. Furthermore, we show that to successfully remove PAH from water, care should be taken for high Hb and ANT concentrations and low H_2O_2 concentrations, otherwise the peroxidase activity of Hb is inhibited by its newly demonstrated catalase activity. Kinetic parameters and optimum pH have been determined for both ANT oxidation (peroxidase activity) and oxygen production (catalase activity).

2 Materials and methods

2.1 Chemicals

Anthracene (ANT) and anthraquinone were purchased from Sigma-Aldrich (99% purity), acetonitrile was of HPLC grade (SDS). Bovine met-Hb was obtained from Sigma-Aldrich as a lyophilized powder (ref H2625). This Hb was used without any purification and considered as a pure preparation with a molecular mass of 64 500 Da. The purity of the preparation was checked using the SDS-PAGE (12% acrylamide) using standard protocol and Coomassie blue staining. No traces of other protein were detected. H_2O_2 was obtained as a 35% solution from Sigma-Aldrich. Chemicals for the buffers were reagent grade (Fluka). The ionic strength of the buffers was 0.05 M. For solutions of pH 3, 4 and 5, a sodium citrate buffer was used ($C_6H_80_7$ / $C_6H_70_7Na$). For buffers of pH 5.5, 6, 7 and 8, a phosphate buffer was used (NaH_2PO_4 / $Na2HPO_4$). All solutions were prepared using de-ionized water from a milli-Q purification system from Millipore.

2.2 ANT oxidation study

ANT oxidation studies were conducted in a 100-mL reaction mixture containing 0.3–15 µM Hb, 0.3–3µM ANT, 1% acetonitrile and 50 mM phosphate buffer at pH 5. ANT oxidation was started by adding various concentration of H_2O_2 (from 0.075 to 3 mM). The reaction progress was followed by ultrafast liquid chromatography (UFLC). ANT disappearance was calculated from the decreasing area of chromatogram peaks between reaction time t = 0 and t = x. For initial rates determination, reactions were stopped at t_x =5 min and the total disappearance measurement reactions were stopped at t_x =15 min. Reactions were performed in flasks protected by aluminum foil to avoid ANT photo-oxidation cross-reaction. To ensure reproducible results, after reaction and before UFLC analysis, samples were diluted in 50% acetonitrile. The dilution step is necessary to stop the reaction and help stabilize the samples before UFLC analysis. All reactions were repeated three times. Blanks without H_2O_2 and without Hb were also run. Oxidation products were identified by GC-MS after a continuous extraction of the media with 200 mL methylene chloride. The organic phase was dried with Na_2SO_4 and concentrated to 1 mL. Anthraquinone production was quantified using a calibration curve prepared with the commercial standard compound.

2.3 UFLC analyses

The UFLC system used is a Shimadzu instrument, which was equipped with a Supelco reversed phase column C18-PAH (50 mm length, 4.6 mm internal diameter, and 3 µM particle size). The chromatographic apparatus is composed of two pumps LC-20AD, an automatic sampler SIL-20AHT, a diode array detector SPD-M20A, a column oven CTO-20A, and a communication bus module CBM-20A. Chromatograms were monitored with LabPower Shimadzu software. The separation method consisted of a gradient between solvent A (50:50 water/acetonitrile) and solvent B (acetonitrile) starting from 0% of B over 0.5 min, then increasing B up to 75% from 0.5 to 3 min, maintaining 75% of B from 3 to 4.2 min, increasing B up to 100% from 4.2 to 4.5 min and maintaining it to 5 min. The column oven was maintained at 40°C during the analysis. The retention time of ANT in this condition was 2.3 min. Peak integration was done at the maximum wavelength adsorption of the compound (251 nm).

2.4 GC-MS analyses

GC-MS analyses were performed with a Shimadzu GC-2010 coupled to a Shimadzu MS-2010 and equipped with a Supelco SPB-5MS capillary column (30 m × 0.25 mm). Ionization was carried out by electronic impact (70 eV). The temperature of the ion source was 200°C and the temperature interface was 280°C. The oven temperature program started with 100°C for 5 min, then increased 5°C/min to 280°C and remaining at 280°C for 11 min. From the extracted and concentrated sample, 1 µL was injected with a split equal to 10 and detection in the mass spectrometer was done in scan mode between 50 and 400 *m/z*. Under these conditions, the retention times of ANT and anthraquinone

were 15 and 17 min, respectively. Compound identification was done with the NIST mass spectral database. The principal *m/z* for anthraquinone were 76, 152, 180, 208 and 178 for ANT.

2.5 Oxygen-production measurement

Oxygen production was measured with an oxygen electrode CellOx 325 coupled to an Inolab 730 oxymeter, both from the WTW Company. The measurement was started by addition of H_2O_2 under soft stirring. The reaction volume was fixed at 100 mL. Oxygen production rates were measured in the first minute of the reaction. The effect of pH and of different H_2O_2 and Hb concentrations was studied. Reactions were repeated three times and blanks without H_2O_2 and without Hb were performed.

2.6 Kinetics parameters determination

The kinetic parameters of the catalase-like activity and ANT oxidation activity of Hb were determined graphically by plotting experimental data obtained by measuring the initial rates of activity (Vi) toward the substrate (H_2O_2, ANT) concentration (S). The different kinetic parameters were determined using the Lineweaver-Burk representation, 1/Vi = f (1/S), *i.e.*, Michaelis constant (K_M), maximum rate activity (V_m) and indirectly the catalytic constant (k_{cat}).

3 Results and discussion

3.1 ANT oxidation through the peroxidase activity of Hb

To determine optimal pH reaction conditions, the peroxidase activity of Hb (0.15 µM) was measured between pH 3.0 and 8.0 in the presence of 150 µM H_2O_2 and 0.3 µM ANT (Fig. 2). The results show a narrow pH range for peroxidase activity, with an optimum pH value between 5.0 and 6.0. At pH 5.0, the maximum peroxidase activity corresponds to 76 ± 5% of ANT elimination. At pH 4.0 and 7.0, the remaining activity is less than 40% and 20% of the maximum activity, respectively. In the following studies, pH was maintained at 5.0.

To be as close as possible to waste-water specificities, the challenge of the present work was to use very low co-solvent (acetonitrile) addition, ~1%, and low ANT concentration (0.3–3 µM). Previously, Vazquez-Duhalt *et al.* [29] had noticed that the biocatalytic oxidation of PAH by Hb and H_2O_2 is strongly dependent on organic solvent concentration with an optimum at 15% acetonitrile. According to this study, decreasing the acetonitrile concentration leads to a serious decrease of activity. For example, in 10% acetonitrile, the relative specific activity of Hb falls to 60%. This may be due either to the fact that, for organic co-solvent concentrations below 10%, the diffusion of PAH to the protein active site is slow, or that amount of co-solvent was not sufficient to dissolve the PAH in water since Vazquez-Duhalt *et al.* used an initial PAH concentration of 30 µM. It should be noted that these concentrations are 100- to 1000-fold higher than the one present in waste water (the goal of our study). As PAH compounds are very hydrophobic, they ex-

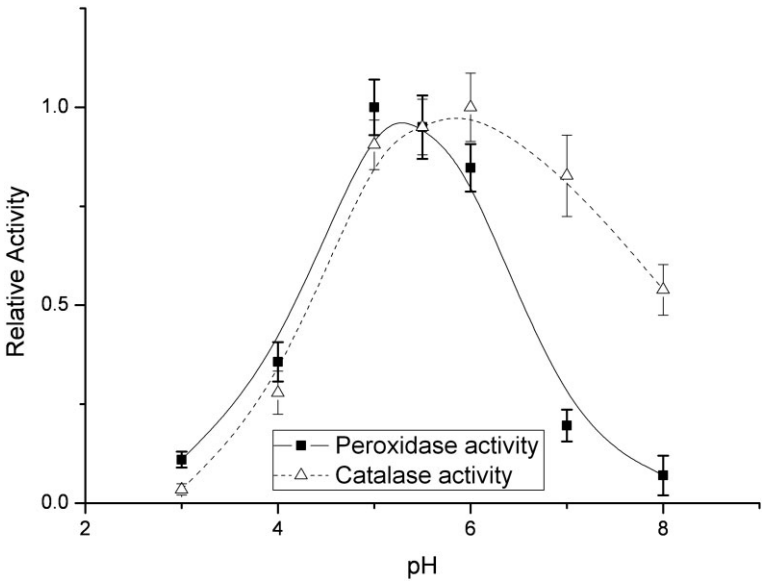

Figure 2. Relative Hb pseudo-peroxidase and catalase activities *versus* pH. For peroxidase-like activity, the initial ANT concentration was 0.3 µM, 1% acetonitrile, [H_2O_2] =150 µM and [Hb] =0.15 µM. The maximum activity, in terms of total ANT disappearance, was 76 ± 5% at pH 5. Analyses were done after complete reaction (15 min) by UFLC. For catalase-like activity, [Hb] =3 µM, [H_2O_2] =5 mM. Analyses were done with an oxymeter during the first few minutes after addition of H_2O_2. The maximum activity, in terms of initial O_2 production rate, was 62 µM/min at pH 6.

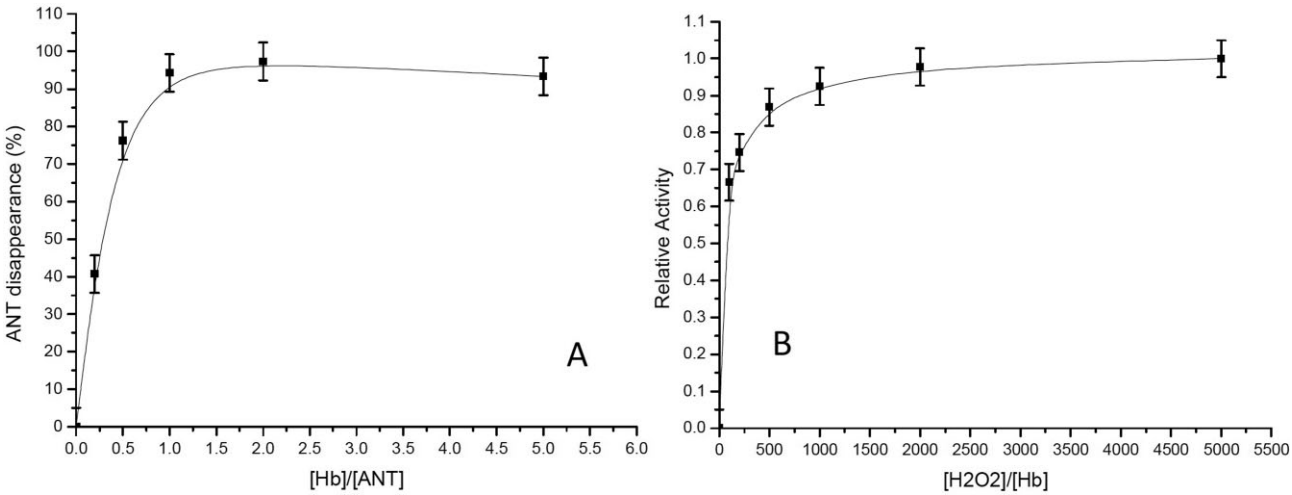

Figure 3. (A) Total ANT disappearance *versus* Hb concentration. The initial ANT concentration was 0.3 μM in 50 mM phosphate buffer, pH 5, 1% acetonitrile. A molar excess $[H_2O_2]/[Hb]$ =1000 was used for all reactions. Analyses were made after complete reaction (15 min) by UFLC. (B) Relative activity of Hb on ANT elimination at variable $[H_2O_2]/[Hb]$ ratios. The initial ANT concentration was 0.3 μM in 50 mM phosphate buffer, pH 5, 1% acetonitrile. Hb concentration was 0.15 μM. Maximum activity, in terms of total ANT disappearance was 85 ± 5% for $[H_2O_2]/[Hb]$ =5000. Analyses were made after complete reaction (15 min) by UFLC.

hibit a low solubility in water. ANT solubility in pure water is 7.3 μM [33]. Furthermore, H_2O_2 is a suicide substrate which inactivates the protein at the same time that it is used for the catalytic reaction [34]. One could think that, for a low acetonitrile content, the inactivation of the protein would be faster than the PAH oxidation because of a better H_2O_2 diffusion.

We have shown that total ANT disappearance, for a concentration in water of 0.3 μM in a medium containing 1% acetonitrile, is possible if a stoichiometric amount or an excess of Hb with respect to ANT is used. Nearly total disappearance of ANT was reached when using a stoichiometric quantity or a twofold molar excess of Hb (94 ± 5% and 97 ± 5%, respectively) (Fig. 3A). Noteworthy, a very large molar excess of H_2O_2 was necessary to reach good levels of ANT oxidation. Indeed, an excess of at least 1000, with respect to Hb concentration, was needed to reach 90% ANT oxidation (Fig. 3B). ANT oxidation, under these conditions, can be qualified as "pseudo-catalytic" because, even if Hb accelerates the reaction (allowing oxidation of ANT in the presence of H_2O_2), excesses of Hb and peroxide are required for a complete disappearance of ANT. One could think that the sub-stoichiometric behavior of the reaction would limit the potential application of the process at an industrial scale but, as bovine met-Hb is a waste product from the food industry, it does not. In fact, abattoirs are an unlimited source of cheap bovine met-Hb. Moreover, the total disappearance of ANT under our conditions is reached in about 15 min, which is much faster than usual catalytic process used to remove PAH. Methods such as oxidation catalyzed by porphyrin [35] or an inorganic mineral oxide catalyst [36] with H_2O_2 or UV take several hours at least to reach their maximal efficiency.

Concerning ANT oxidation products, a small peak was observed using the UFLC technique corresponding to 35% of the ANT that had disappeared. It was identified as anthraquinone by GC-MS. The other remaining 65% is supposed to be covalently linked to Hb as previously shown for other PAHs [37, 38]. In the present study, we use the term 'disappear' to qualify the reaction of Hb and H_2O_2 over ANT, because only a part of the disappearing substrate has been clearly identified as an oxidized product, and further studies are necessary to determine the exact state of ANT linked to the protein and its localization on the protein. This could be performed using [^{14}C]ANT followed by the entire enzymatic digestion of the reacted Hb to identify the region of the Hb to which the ANT is attached, or by analyzing oxidized PAH released after total hydrolysis of Hb, as shown by Melikian *et al.* [39]. It is probable that the ANT is semi-oxidized as no adsorption of ANT and anthraquinone onto the protein was observed while running blank reactions without H_2O_2. Considering this fact, the term 'oxidation' can also be employed to qualify the reaction of ANT with Hb and H_2O_2. It should be noted that if the cross-linking of the ANT oxidation product to the protein is verified, it would also be an advantage for industrial processes, because a major part of the oxidation product is trapped with-

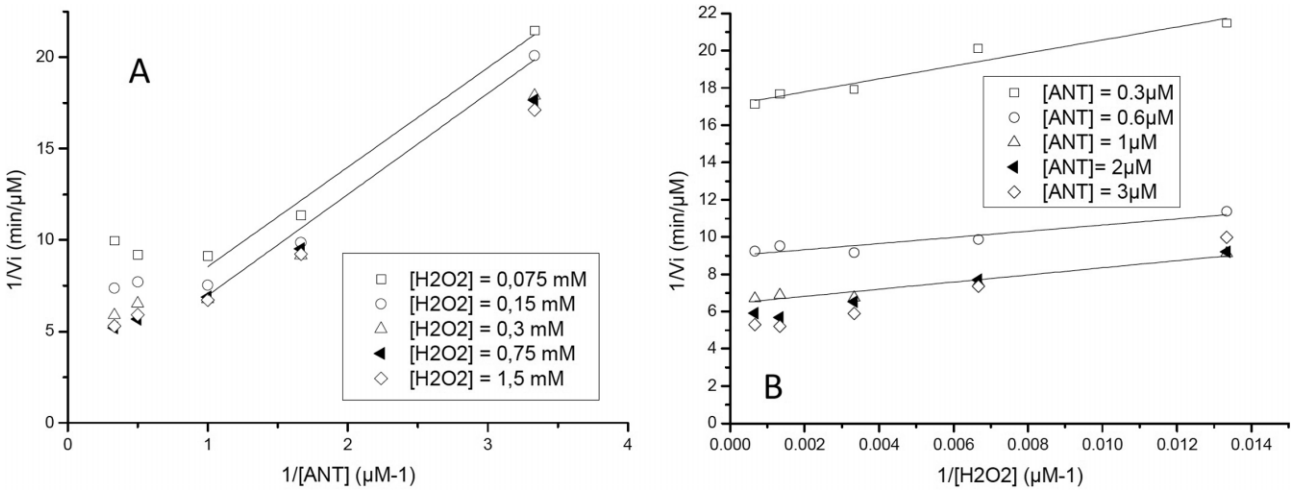

Figure 4. Lineweaver-Burk plot of initial ANT disappearance rates, at variable H_2O_2 concentration (A) and at various ANT concentrations (B). Reactions were conducted in 50 mM phosphate buffer pH 5, with different H_2O_2 concentrations (0.075, 0.15, 0.3, 0.75, 1.5 mM), various ANT concentrations (0.3, 0.6, 1, 2, 3 µM) and a fixed Hb concentration (1.5 µM). Analyses were done after 5 min of reaction by UFLC.

in the protein. If Hb is immobilized on a support, a recovery of the oxidized PAH/protein system is possible as well as its elimination by a simple calcination process. Even if the oxidized forms of PAH are more biodegradable, they are also more carcinogenic [40] and as such, this global elimination process has to be considered.

To understand the mechanisms of ANT oxidation by Hb and to measure kinetic parameters, initial rates of the reaction (Vi) were assayed for different ANT concentrations (0.3, 0.6, 1, 2, 3 µM), various H_2O_2 concentrations (0.075, 0.15, 0.3, 0.75 and 1.5 mM) and fixed Hb concentration (1.5 µM). The Lineweaver-Burk representations, $1/Vi = f(1/[ANT])$ and $1/Vi = f(1/[H_2O_2])$ (Fig. 4) show parallel lines for low ANT and high H_2O_2 concentrations, which means that the oxidation of ANT by Hb fits a bi-bi ping-pong mechanism. This mechanistic model is typical of the peroxidase group [41, 42]. First, H_2O_2 binds to the porphyrin iron to be activated, and then ANT reacts with the activated oxygen species. Nevertheless, for high ANT and low H_2O_2 concentrations, the lines are no longer parallel, showing a slowing of the ANT disappearance rates. This is not easy to observe in Fig. 4 because the rates are very close to one another, due to the tight differences between the various ANT concentrations studied, but for H_2O_2 ≤0.15 mM and ANT ≥2 µM the rates of reaction decrease.

Kinetic parameters determined for ANT oxidation were V_{max}=0.25 ± 0.01 µM/min, $K_{M(H2O2)}$ =80 µM, $K_{M(ANT)}$ =1.1 µM and k_{cat} =0.170 ± 0.005/min. The very tight K_M for ANT is surprising, but it should be noted that it was not determined under saturation conditions. Indeed, due to its poor solubility in an aqueous medium, the maximum concentration of ANT was 3 µM far below Hb saturation ([Hb] =1.5 µM). Moreover, the Hb concentrations required to accurately measure oxidation rates need to be high compared to the ANT concentrations. Thus, it was not feasible to work with an excess of ANT in a medium containing only 1% acetonitrile. Nevertheless, our data shows that the system works almost as if it was saturated with ANT. With saturating H_2O_2 concentrations, the initial rates over 1 µM ANT, increased just slightly (Fig. 4). Considering this fact, our results showed that the reaction was slow (V_{max} =0.25 ± 0.01 µM/min) but this was compensated by the good affinity of ANT for Hb ($K_{M(ANT)}$ =1.1 µM). The observed rate (0.17/min) in our study is below that reported by Vazquez-Duhalt and colleagues in 1995 and 2000 (0.4/min) [29, 31]. Firstly, this difference can be explained by the co-solvent concentration. In our study, 1% acetonitrile was used in contrast to 15% in this previous work. More acetonitrile in the media can increase the ANT solubility with an impact on the kinetic parameters. On the other hand, Vazquez-Duhalt and colleagues worked with human Hb, which can have a slightly different reactivity. However, even if both proteins react in exactly the same way, the purification process to obtain the proteins can vary, and bovine met-Hb, even if as pure as human Hb, can be less active. Whatever the case, our approach is more suited to a possible industrial waste-water biotechnological treatment processes because of the reactions conditions (low co-solvent and PAH concentrations) and because

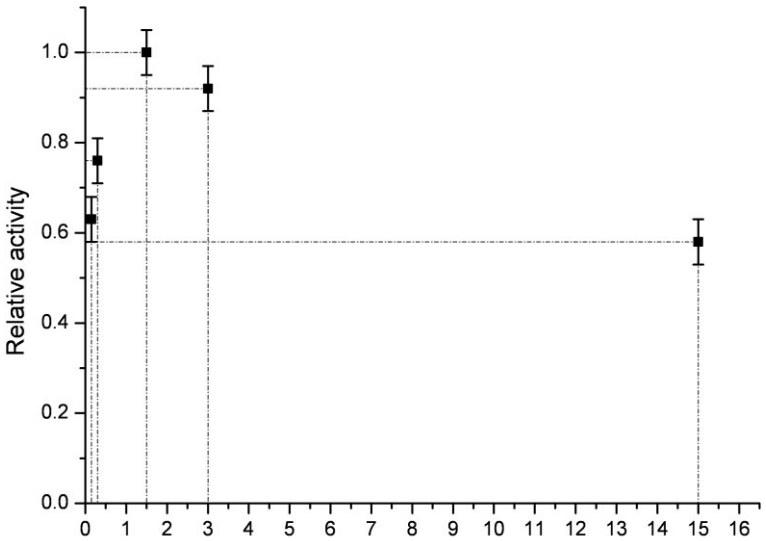

Figure 5. Relative peroxidase-like activity of Hb on ANT oxidation at low H_2O_2 concentration (150 µM) in function of Hb concentrations. Reactions were done in 50 mM phosphate buffer, pH 5, [ANT] =3 µM. The maximum activity, in term of total disappearance, was 57 ± 5% with [Hb] =1.5 µM. Analyses were done after complete reaction (15 min) by UFLC.

bovine met-Hb is more suitable than human Hb for a biotechnological process.

Working with a fixed and low H_2O_2 concentration (150 µM), variable Hb concentrations (0.15, 0.3, 1.5, 3, 15 µM) and high ANT concentration (3 µM) (Fig. 5), in the zone where ANT oxidation rates were slow down (Fig. 4), showed that this inhibition is not due to a typical substrate-excess inhibition. On one hand, Hb peroxidase relative activity (in terms of total ANT disappearance) increased from 63 to 100% between 0.2 and 1.5 µM Hb. On the other hand, the same relative activity falls from 100 to 58% between 1.5 and 15 µM Hb. To summarize, considering results from the Figs. 4A and B, discussed above, the inhibition of ANT oxidation takes place for $[Hb]/[H_20_2] \leqslant 100$ and $[H_2O_2]/[ANT] \leq 75$. These results define an inhibition zone that should be taken into account for any likely industrial application for PAH removal in aqueous media. Nevertheless, reaction conditions are usually outside of this inhibition zone because a large excess of peroxide compared to Hb is used and the PAH concentration in waste water is very low (nM).

These results suggested that H_2O_2 was consumed through an alternative pathway. Additionally, for [Hb] =15 µM and $[H_2O_2]$ =15 mM, small gas bubbles appeared and foam formed. This observation strongly suggested that Hb produced a gas

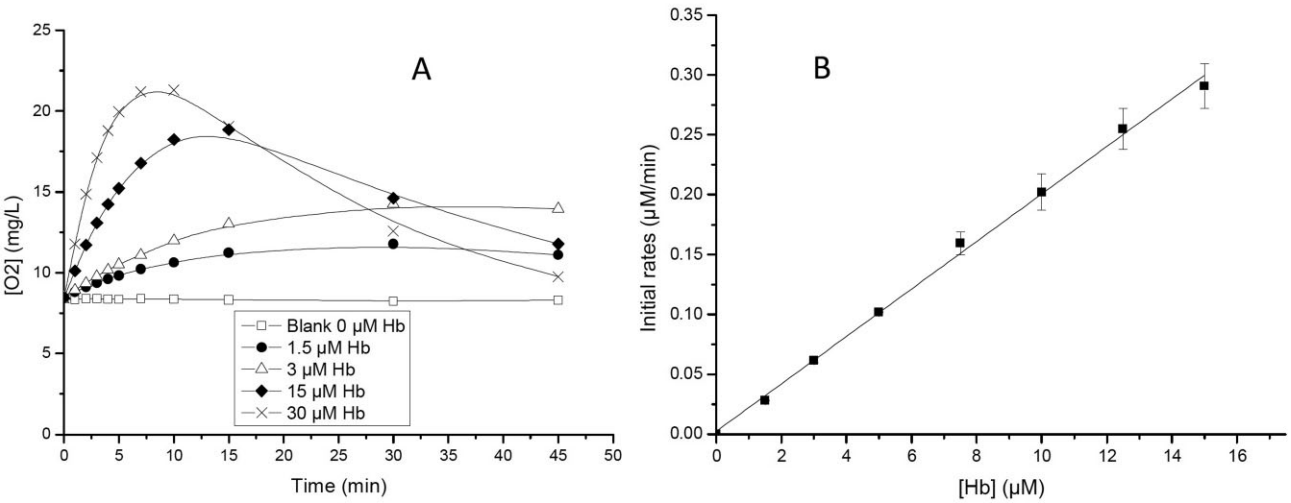

Figure 6. (A) O_2 emission (mg/L) for $[H_2O_2]$ =1.5 mM and various Hb concentration (0, 1.5, 3, 15, 30 µM). Measurements were done with an oxymeter. (B) Linear initial O_2 production rates in function of Hb concentration (1.5, 3, 5, 7.5, 10, 12.5, 15 µM). H_2O_2 concentration was kept fixed for all reactions (5 mM). Reactions were done in 50 mM phosphate buffer, pH 6. Measurements were done with an oxymeter in the first few minutes after H_2O_2 addition.

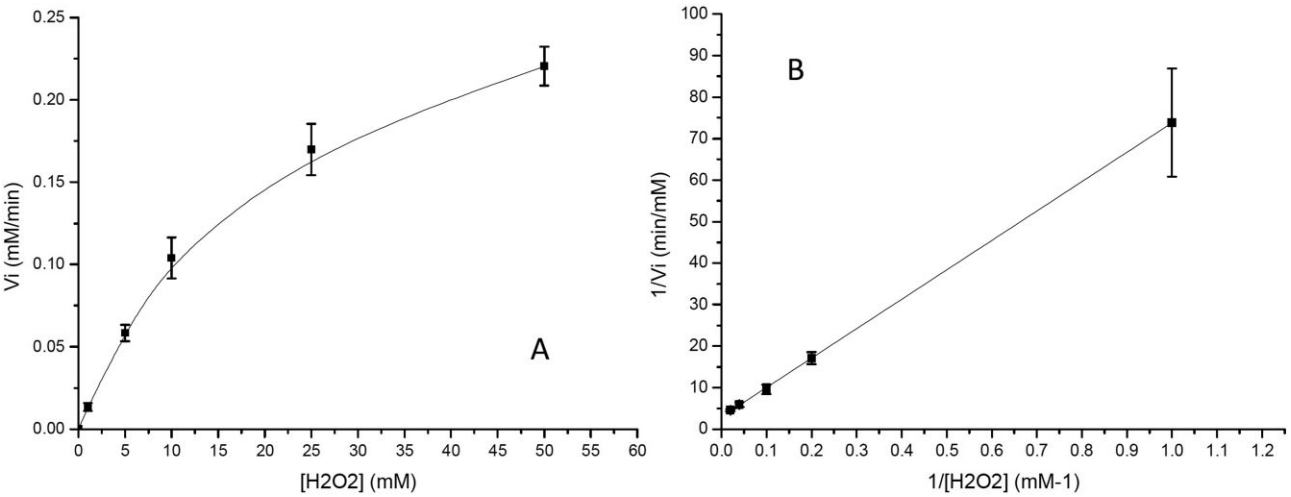

Figure 7. (A) Initial O_2 production rates by Hb in function of [H_2O_2] showing the saturation kinetic of Hb catalase activity. Reactions were done in 50 mM phosphate buffer, pH 6, [Hb] =3 µM. Measurements were done with an oxymeter during the first minutes after H_2O_2 addition. (B) Lineweaver-Burk plot of initial O_2 production rates.

from H_2O_2. The sole gas that could potentially be produced by hemoproteins is O_2, coming from the dismutation of H_2O_2 through a catalase-like mechanism. During this type of activity H_2O_2 plays both an oxidative and a reductive role to form molecular oxygen [14] (Fig. 1). However, this reaction has never been observed for Hb.

3.2 Catalase-like activity of Hb

To study the potential catalase-like activity of Hb and to ensure reliable results, the following experiments were performed in absence of ANT. A high concentration of H_2O_2 was used to have significant O_2 production rates and O_2 emission was measured by oxymetry.

Initially, optimal pH reaction conditions were determined by measuring the catalase activity between pH 3.0 and 8.0 with 3 µM Hb in the presence of 5 mM H_2O_2 (Fig. 2). Results showed a wide pH range for O_2 emission. The optimum pH activity was between pH 5.0 and 7.0, but even at pH 8.0, more than 50% of Hb catalase-like activity remained. The catalase activity of Hb was less sensitive to pH compared to its peroxidase activity. It should be noted that at pH 8, only catalase activity occurred, but there was no pH value at which one can work with only the peroxidase activity of bovine met-Hb. The following experiments were run at pH 6.0.

For [H_2O_2] =1.5 mM, increasing the Hb concentrations (1.5, 3, 15, 30 µM) led to an effective production of O_2 with regards to Hb concentration. Whereas the solubility of O_2 in water is only 8 mg/L, an emission of 22 mg O_2/L has been measured in 10 min with 30 µM Hb (Fig. 6). The O_2 production rates evolved linearly (r^2 =0.9971, Fig. 6) clearly demonstrating the catalytic nature of this catalase-like activity. Initial O_2 production rates were determined for 5 mM H_2O_2 and various Hb concentrations (1.5, 3, 5, 7.5, 10, 12.5, 15 µM). For a fixed H_2O_2 concentration, more Hb gave more O_2 production.

The Lineweaver-Burk representation of O_2 production (Fig. 7), measured at a fix Hb concentration (3 µM) *versus* increasing H_2O_2 concentrations (1, 5, 10, 25, 50 mM), was used to determine the kinetic constants of Hb catalase-like activity. These were V_{max} =344 ± 24 µM/min, K_M =24.4 ± 5.2 mM and k_{cat} =115 ± 8/min. These values show that Hb catalase-like activity is fast but the affinity of H_2O_2 for Hb is weak. In term of comparison, Hernandez-Ruiz *et al.* [17] calculated the catalytic parameters for the catalase-like activity of HRP and found a K_M =4 mM and k_{cat} =110/min. The kinetic parameters of the catalase-like activity of chloroperoxidase from *C. fumago* measured by Sun *et al.* [16] were K_M =3.3 mM and k_{cat} =54 000/min. The catalase activity of Hb was faster but in the same range of order as HRP, but much slower than chloroperoxidase. However, the affinity of H_2O_2 is lower for Hb than for HRP and chloroperoxidase, which are equal to each other.

An explanation for this catalase activity could be the presence of a contaminating catalase in the met-Hb preparation. However, this hypothesis can be easily contradicted since the kinetic parameters of Hb catalase activity are very different from those found for real catalases, such as catalase from *Mi-*

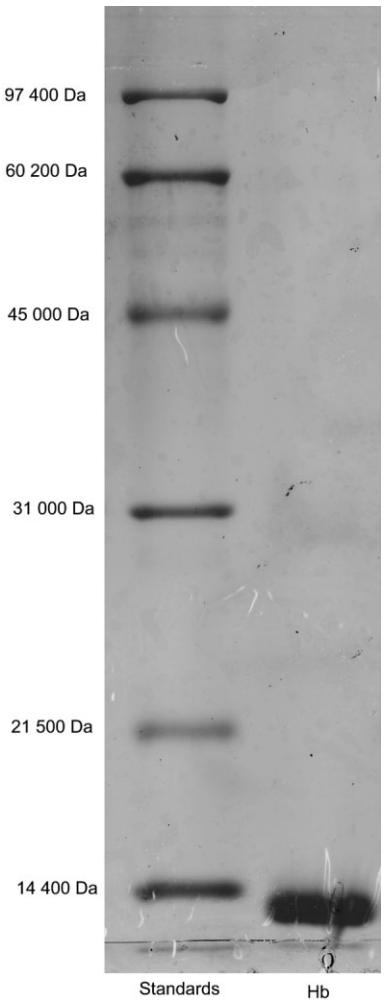

Figure 8. SDS-PAGE gel photo. Left: Standards with various molecular weights. Phosphorylase b (97 400 Da), bovine serum albumin (66 200 Da), ovalbumin (45 000 Da), carbonic anhydrase (31 000 Da), soybean trypsin inhibitor (21 500 Da) and lysozyme (14 400 Da). Right: Hb (10 g/L).

crococcus lysodeikticus [43, 44], featuring a K_M of 1100 mM for H_2O_2, which is almost 46-fold higher than the one calculated for Hb (25 mM). Additionally, the Hb catalase activity presents a saturation kinetic under steady-state conditions, which is not the case of the bacterial catalase (Fig. 7). These kinetic elements are sufficient to show that Hb preparation is not contaminated by catalase. Two different batches of Hb were used with two commercial purities (Sigma-Aldrich, cat. nos. H 2625 and 2500), and the same catalase activity was found. Moreover, SDS-PAGE of the Hb indicated no traces of other proteins, showing that this activity does not come from some contaminating residual enzymatic activity (Fig. 8). Nevertheless, as this Hb catalase-like activity has never been described before, it should be verified in further studies to see whether it also exists for human and other Hb proteins.

The Hb catalase activity described here can be defined as a promiscuous catalytic activity [45] since it is a side reaction catalyzed by the wild-type Hb, which is distinctly different from the main function known for Hb. This "promiscuous" property is thought to be an evolution-related process [46], allowing organisms to survive under changing conditions [47]. Thus, it could be assumed that this Hb catalase activity is the legacy of natural evolution, from a common proteic ancestor of hemoproteins that has differentiated progressively one from the other, creating several different functions from a common active site. A few structural mutations induced during this time could have given some hemoproteins either an enzymatic activity like peroxidases, catalases or oxygenases, or others the ability to transport small molecules. The presence of significant or weak promiscuous activities widens the biotechnological applicability of Hb, and makes the hemoprotein superfamily a very interesting group for structure-activity relationship studies. Some researchers have already underlined common structural elements between these different classes of enzymes [48, 49] and further studies, including studies of Hb, should yield important information for understanding how the same global structure can lead to different activities. For example, Jakopitsch and colleagues [50, 51] have shown that some distal amino acids such as Asp152 and Trp122 are essential for the catalase activity, but not for the peroxidase activity of the bifunctional KatGs. Bovine met-Hb displays a totally different heme distal environment, which could lead to other conclusions concerning the origin of catalase activity.

In addition, it is possible that met-Hb, which cannot bind O_2, could act *in vivo* as a protective protein against H_2O_2 accumulation. Indeed, H_2O_2 is constantly produced from oxygen active species in erythrocytes [52, 53]. Thus, it could be transformed to O_2 through met-Hb catalase activity. On this support, it can be supposed that a slight mutation modifying this catalase-like activity of Hb could have important consequences *in vivo, e.g.*, for lipid and lipoprotein peroxidation [54–56].

Concerning PAH removal from industrial waste water, when using the peroxidase activity of Hb, the inhibition of the ANT disappearance rates can be eliminated by avoiding the inhibition zone described above, but the catalase-like activity will always run in parallel of the peroxidase activity, consuming some H_2O_2 molecules.

4 Concluding remarks

ANT oxidation by the peroxidase-like activity of Hb proceeds through a bi-bi ping-pong mechanism. In aqueous medium, ANT oxidation rates and turnover numbers are very low, probably due to the limited diffusion of ANT to the active site of Hb. It can be qualified as a pseudo-catalytic system. In aqueous media, total disappearance of ANT traces can be performed within 15 min but with high amount of Hb. This requirement does not prohibit industrial applications because bovine met-Hb is a waste from food industry with a low production cost.

Bovine met-Hb presents a catalase-like activity, described here for the first time. This activity interfered with the peroxidase-like activity especially for $[Hb]/[H_20_2] \leq 100$ and $[H_2O_2]/[ANT] \leq 75$.

Thus, as recently defined [44], Hb is a good example of accidental catalytic promiscuity. Despite the fact that its principal role is to transport oxygen, it also possesses low peroxidase-, oxygenase- and catalase-like activities.

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