ASCORBATE BINDING TO GLOBINS

FLORINA-VIOLETA DEAC, ANAMARIA TODEA, ANA MARIA BOLFA, PAULA PODEA, PETRONELA PETRAR, RADU SILAGHI-DUMITRESCU

Department of Chemistry and Chemical Engineering, “Babes-Bolyai” University, 11 Arany Janos Str., Cluj-Napoca RO-400028, Romania

(Received August 25, 2009)

In agreement with data known for other globins, bovine hemoglobin (Hb) is found to catalyze peroxide reduction by ascorbate, with a relatively low $K_m$ for ascorbate. NMR spectra directly demonstrate the existence of an Hb-ascorbate complex in solution; serum albumin is also shown to bind ascorbate, suggesting that, whether inside or outside of the red blood cell, ascorbate is most likely predominantly found as a complex with a protein such as hemoglobin and albumin.

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

INTRODUCTION

The general antioxidant properties of ascorbate and in particular its role in protecting blood from oxidative stress are well known (1–6). 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 (4, 7). Human hemoglobin and horse heart myoglobin were both shown to act.

As ascorbate peroxidases, with well-measurable Michaelis-Menten parameters, which included $K_m$ values close to physiological ranges (4). Here, the ascorbate peroxidase activity of bovine hemoglobin is described, also featuring a very low $K_m$ value for ascorbate. Consistent with this low $K_m$, NMR spectra demonstrate an adduct between ascorbate and hemoglobin; furthermore, ascorbate forms adducts with serum albumin, suggesting that in vivo ascorbate will always be found predominantly bound to proteins.

* Corresponding author (Email: rsilaghi@chem.ubbcluj.to)

ROM. J. BIOCHEM., 46, 2, 115–121 (2009)
MATERIALS AND METHODS

Bovine hemoglobin was purified following a variation of the general protocol of Antonini and Brunori (8). Bovine blood, freshly drawn on citrate, was centrifuged 15 minutes at 5000 rpm (g) 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. Myoglobin (lyophilized from horse heart) and bovine serum albumin (fraction V) were purchased from Sigma and used without any further purification. The met forms of hemoglobin and myoglobin were prepared by ferricyanide treatment as previously described (1, 9, 10).

Ascorbate peroxidase activity was measured monitoring the time course of absorbance at 290 nm (specific for ascorbate), under conditions detailed in Figure legends (4, 11). At this wavelength there are no significant changes in absorbance assignable directly to protein or hydrogen peroxide during the experiments. The presence of dehydroascorbic acid as primary reaction product was not verified directly, but it was inferred by similarity with previously reported data on heme-containing ascorbate peroxidases (11, 12).

UV-vis spectra were recorded on Agilent 8453 (Agilent, Inc.) and Cary 50 (Varian, Inc) instruments. NMR spectra were recorded in D2O, pH=7.4 phosphate buffer on a 300MHz Bruker Avance DPX-300 instrument, at a protein concentration of 25 mg/mL; sample preparation and data collection typically took no more than 30 minutes, to avoid any measurable changes in sample composition either via reduction of the iron by ascorbate or via ascorbate auto-oxidation.

RESULTS AND DISCUSSION

Figure 1 shows turnover data for peroxide reduction by ascorbate as catalyzed by bovine hemoglobin (bHb), in a peroxidase-type reaction where ascorbate and peroxide bind sequentially to the protein. The data indicates that bHb catalyzes ascorbate oxidation by hydrogen peroxide, in a manner very similar to those previously demonstrated for human hemoglobin and horse heart myoglobin (4). Thus, $K_m$ and $k_{cat}$ parameters for ascorbate as well as for peroxide are measurable and indicate, as in the case of human Hb, a low $K_m$ for ascorbate, at 21 µM(4). On the other hand, the $K_m$ for peroxide is calculated from Figure 1 to be 1630 µM, in line with parameters of similar magnitude reported for bona fide peroxidases (4, 13). $k_{cat}$ values computed for ascorbate and peroxide, respectively, are slightly different: 73 s⁻¹ based on peroxide and 24 s⁻¹ based on ascorbate, which should not be the case for a true two-substrate reaction mechanism. This difference is due to the fact that ascorbate dependence experiments (Fig. 1B) were conducted
at 800 µM peroxide, which is not saturating with respect to peroxide; however, at higher peroxide concentrations the direct reactions between peroxide and ascorbate would prevent reliable measurements of ascorbate peroxidase activity. Our unpublished data show that several other globins of animal origin, some of which have higher affinities for peroxide than bovine Hb, all feature similarly low apparent $K_m$'s for ascorbate. The actual value of this $K_m$ is expected to be higher than the 21 µM measured here under non-saturating peroxide, but still in close range of the physiological concentrations of ascorbate in the blood (50–200 µM) (1).

Fig. 1. – Dependence of bovine hemoglobin-catalyzed ascorbate consumption rate on peroxide (panel A) and ascorbate (panel B) concentration, respectively. At pH 7.4, 50 mM phosphate, 10 µM Hb, room temperature; top panel-400 µM ascorbate; bottom panel –800 µM peroxide. The curves show fits of experimental data to Michaelis-Menten kinetics as detailed in Materials and Methods.
In vivo, hemoglobin and ascorbate share the same compartment (the red blood cell, at concentrations far exceeding those under which the $K_m$ measurements of Fig. 1 were performed: millimolar amounts for Hb and 50–200 µM for ascorbate (1). It follows (with the caveat that $K_m$ being a kinetic parameter is not truly a binding constant) that one might expect most of the erythrocyte ascorbate be bound to hemoglobin in vivo; however, to our knowledge no direct evidence for a stable ascorbate-hemoglobin complex is available to date. Figure 2 shows proof of such a complex: NMR spectra are shown of ascorbate in the presence and absence of bHb at neutral pH and with the two partners present in equimolar amounts. Signals belonging to ascorbate protons bound to carbon atoms 5 and 6 (CHOH and CH₂OH, sugar nomenclature) are easily observable at 4 ppm and 3.6 ppm, respectively; these signals are seen to shift and change intensity in the presence of bHb, which

---

**Fig. 2.** – NMR spectra of ascorbate, met hemoglobin and an equimolar ascorbate-met hemoglobin mixture. UV-vis spectra (not shown) indicate no reduction of the heme iron within the time course of the experiment. NMR spectra using cyanomet-Hb (not shown) show that signals assignable to the heme protons, detectable at 15–30 ppm, are not affected by the presence of ascorbate.
can only be interpreted to mean that a direct, stable interaction occurs between Hb and ascorbate. This offers a confirmation for the hemoglobin:ascorbate adduct inferred from the peroxidase-type kinetics. The different response to Hb of the two types of protons in ascorbate (on carbon 5 and carbon 6) suggests that carbon 6, which is further away from the furanose-type ring, has less interaction with the protein as compared to carbon 5. We propose that binding involves an ionic interaction between the deprotonated furanose ring of ascorbate and lysines located on the surface of the protein. Previous kinetic, spectroscopic and site-directed mutagenesis studies on ascorbate-globin interaction have identified tyrosine 42 on the alpha subunit as the most likely site for ascorbate reaction with the high-valent ferryl form of hemoglobin (14). Lysine 90 (α chain) and arginine 40 (β chain, human Hb numerotation), found nearby Tyr42 on the outer surface of the protein, are likely candidates for defining an ascorbate-binding site in globins. An alternative location for an ascorbate binding site, albeit not in the immediate location of Tyr42, would be the effector binding pocket defined at the junction between the four subunits, which in all globins features positively-charges side-chains (15).

Figure 3 shows that ascorbate also binds to serum albumin; while the C5 and C6 protons are again involved, the sign and size of the changes in signal positions differ clearly from the bHB-ascorbate complex together with the bHb data. Fig. 3
suggests that ascorbate is mostly found bound to proteins in red blood cells as well as in the surrounding plasma. In light of the different binding modes of ascorbate to the two proteins examined here, it is expected that similar experiments may uncover binding phenomena with several other proteins. Further studies are underway to characterize in more detail these binding phenomena and their biological relevance.

In conclusion, ascorbate has been shown to bind to blood proteins hemoglobin and albumin; the relatively low $K_m$ estimated for the hemoglobin-ascorbate interaction places physiological relevance upon these data.

Acknowledgements: Financial support from the Romanian Ministry of Education and Research (grant PNII Idei 565/2007) is gratefully acknowledged.

REFERENCES


