# Reactions of Ascorbate and Hydrogen Peroxide with Hemocyanin from Taiwan Snails and Comparison with a Synthetic Dicopper(I) Oxygen Carrier

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(Received May 6, 1987)

Hemocyanin (Hc) from Taiwan snails (*Achatina fulica*) was prepared and purified in the manner previously described [1]. Cu can be removed from Hc by KCN treatment [2] and Cu(I) reincorporated to form reconstituted Hc [3]. The reconstituted Hc was dialized against 0.025 M EDTA in 0.1 M acetate buffer, pH 5.7 in $N_2$ atmosphere and then against the acetate buffer alone. The native Hc has a Cu content of 0.24%. After reconstitution, a Cu content of 0.21% was found. The active site in oxyhemocyanin ($HcO_2$) has two copper(II) ions and one dioxygen in the peroxide state [1, 4]. Methemocyanin (metHc) was prepared by dialyzing oxyhemocyanin solution against 0.1 M acetate buffer, pH 5.0, containing 0.025 M azide for 48 h at 37 °C and afterwards against 0.1 M acetate buffer, pH 5.7, for 2 days [5]. As a model for the active site of Hc, we have prepared $Cu_2(EDTB)(ClO_4)_2$ where EDTB is *N,N,N',N'*-tetrakis(2-benzimidazolylmethyl)-1,2-ethanediamine, as described by Wang *et al.* [3]. UV–Vis spectra were obtained on a Perkin-Elmer Lambda 5 spectrophotometer. NMR spectra were obtained on a Jeol FT-100 NMR spectrometer. The observed linewidth was measured from the full linewidth at half amplitude of the peak, as described previously [1].

## Results and Discussion

Addition of ascorbate to oxyhemocyanin results in lowering of the absorbance at 345 nm. The extent of lowering increases with an increase in the ascorbate concentration and with pH. The faster decrease of $A_{345}$ with an increase in pH reflects a faster reaction of ascorbate with dissolved dioxygen, thus lowering $p(O_2)$. For a solution 0.18% $HcO_2$ and 0.01 M ascorbate at pH 9.0, an initial rapid drop in $A_{345}$ occurs. A minimum is seen with $-\Delta A = 0.6$ after 20 min, followed by an increase in absorbance. After 24 h, the absorbance becomes even greater than before the addition of ascorbate. The rise in $A_{345}$ is explained by the formation of hydrogen peroxide from ascorbate and a secondary reaction involving the hydrogen peroxide and an amino acid residue near the active site of the protein. The rise in $A_{345}$ after the minimum at 20 min occurs at pH 8.5 also, but the rise is far less dramatic than at pH 9.0.

For the reconstituted hemocyanin, similar results are obtained, except that the effects of adding ascorbate and of the hydrogen peroxide formed from the ascorbate on $A_{345}$ are smaller than for native Hc. Thus at pH 9.0, the minimum is at $-\Delta A = 0.3$ after 20 min, which is only half of that observed for native Hc. This is accounted for by the smaller number of $CuO_2Cu$ groups in the reconstituted oxyhemocyanin, compared to native Hc (Cu contents of 0.21% and 0.24%, respectively).

Chen *et al.* [1] have shown that the $^{19}F$ NMR linewidth on addition of KF to oxyhemocyanin was increased because the two copper ions in the active site are Cu(II). We have seen that the addition of ascorbate causes a lowering of $p(O_2)$ and absorbance at 345 nm. Even though the ascorbate is probably too large to reach the active site of hemocyanin [6], the lowering of $p(O_2)$ in the presence of ascorbate is accompanied by a change of oxyhemocyanin to deoxyhemocyanin, so that the two coppers become Cu(I). For a solution containing 3% $HcO_2$, 0.2 M KF and 0.05 M ascorbate at pH 9.0, with the $^{19}F$ linewidth of 9 Hz, at $t = 0$, the linewidth decreased to 3 Hz in 20 min and to 1.7 Hz in 90 min. These results lead us to suggest that the oxyhemocyanin, with Cu(II), is being changed to deoxyhemocyanin, with Cu(I).

The regeneration of gastropodan methemocyanin with $H_2O_2$ has been reported [7, 8]. The reaction is probably

$$(Cu^{II}\cdots Cu^{II}) + H_2O_2 = (Cu^{II}(O_2^{2-})Cu^{II}) + 2H^+ \quad (1)$$

and therefore occurs to a greater extent at higher pH. For *A. fulica* methemocyanin, our findings are summarized in Fig. 1. The extent of the reaction is greater at the higher pH of 8.5, compared to pH 5.5, and the extent increases with an increase in $R$ (the molar ratio of $H_2O_2$/Cu). Addition of ascorbate to methemocyanin also raises $A_{345}$, because of the product formation of hydrogen peroxide in the autoxidation of ascorbate. However, the $A_{345}$ increase is far less than that caused by the direct addition of $H_2O_2$. Groeseneken *et al.* [6] have previously reported that it is through the formation of $H_2O_2$ in autoxidation that ascorbate is able to regenerate methemocyanin.

Reedijk *et al.* [9] and other investigators [3] have discussed the importance of $Cu_2(EDTB)^{2+}$ as a

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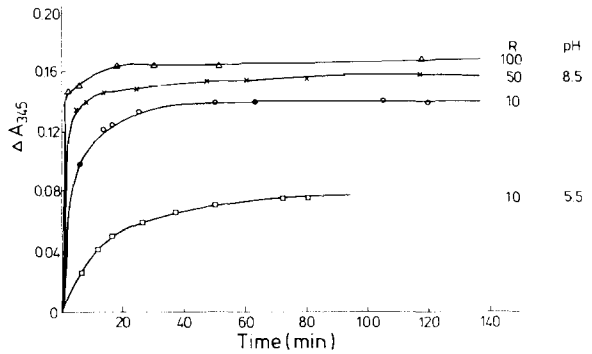


Fig. 1. $\Delta A_{345}$ *vs.* time plots for solutions containing 0.1% methemocyanin, $H_2O_2$ ($R$ = 10, 50 and 100), pH 8.5; and for $R$ = 10 and pH 5.5 ($R$ = $H_2O_2$/Cu).

potential model for the active site of hemocyanin, where EDTB is *N,N,N',N'*-tetrakis(2-benzimidazolylmethyl)-1,2-ethanediamine). The colorless solution of $Cu_2(EDTB)(ClO_4)_2$ in $Me_2SO$ showed an uptake of dioxygen of 0.96 mol/$Cu_2$ [9], resulting in a green solution with maximum absorption at 690 nm, characteristic of Cu(II). The addition of ascorbic acid to the green solution results in a decreased absorbance at 690 nm and exposure to dioxygen raises the absorbance. The effect of adding ascorbate to oxyhemocyanin is also a decrease in absorbance, except that the absorption maximum in the protein occurs at 345 nm. The protein also shows an uptake of 1 mol $O_2/Cu_2$.

$HcO_2$ is ESR-silent because of antiferromagnetic coupling between the two Cu(II) ions, whereas $Cu_2(EDTB)(ClO_4)_2$ in $Me_2SO$, after exposure to air (green solution), gives distinct ESR signals showing two Cu(II) species. In this research we find that the addition of ascorbic acid (0.0031 M) to the green solution (0.0031 M) causes the lower-field Cu(II) signal (see Fig. 2, ref. 3) to disappear, leaving the higher-field Cu(II) signal intact. This leads us to suggest that in the green solution one of the two coppers is more reactive than the other towards ascorbic acid. Beltramini *et al.* [10] have reported that in the reaction between $CN^-$ and Hc, one of the two coppers is also more reactive than the other towards $CN^-$. Thus, there are certain similarities and dissimilarities between the dicopper EDTB compound and hemocyanin.

## Acknowledgements

This work was supported by the National Science Council, ROC. The authors thank Professor R. Lontie for helpful discussion.

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