Nitrite Reductase Activity of Deoxy *Carcinus maenas* Hemocyanin: Formation of the Half-Met Derivative

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The reaction of nitrite with the binuclear copper protein hemocyanin (Hc) leads to the formation of a partially oxidized (half-met) $Cu^{I}-Cu^{II}Hc$, or fully oxidized (met) $Cu^{II}-Cu^{II}Hc$, where the oxidation state of the protein derivative depends on its animal source and the reaction conditions employed.[1−6] Nitric oxide (NO) is another product of this reaction.[4,5] In the presence of excess nitrite, a green half-met (GHM) derivative, in which nitrite is bound to Cu(II),[2,6] is produced in molluscan Hcs as well as in the arthropodan Hc from *Carcinus maenas*. When *Astacus leptodactylus* (arthropoda) Hc is reacted with excess nitrite, met-Hc is formed, together with two equivalents of NO[4] consistent with two, single-electron steps during oxidation of the protein. When deoxy *Helix pomatia* (mollusca) Hc is reacted with excess nitrite, it is suggested that the initial product is also met-Hc,[5] which reacts again with nitrite to yield GHM-Hc. In order to initially form met-Hc through a two-electron oxidation of deoxy Hc, the chemistry requires that 2 equiv of NO are formed. Only one was found.

In this note, we establish that where nitrite is the limiting reagent, an obligatory met intermediate is not formed in the case of *C. maenas* Hc. Thus, the reaction can be written as in Scheme 1 where nitrite is a single electron oxidant of the binuclear active site. The proposed reaction scheme is based on a material balance of reactants and products and is in accord with the observation of a pH dependence for GHM−Hc formation using excess nitrite[4,7] and with the identification of NO formed in the reaction.[4,5]

Scheme 1

$$Cu^{I}-Cu^{I}Hc + HNO_2 + H^+ \rightarrow Cu^{I}-Cu^{II}Hc + NO + H_2O$$

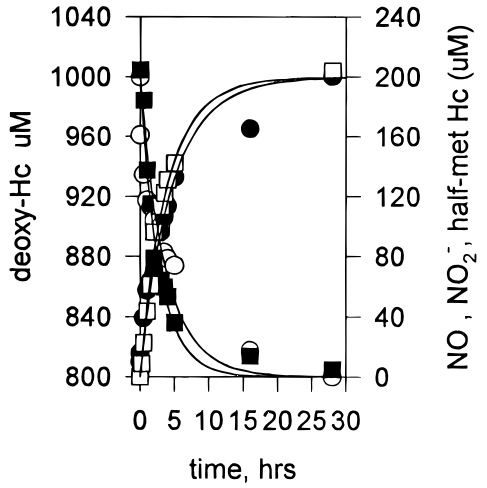

Figure 1. Time course of the reaction of nitrite with deoxy *Carcinus maenas* Hc: (○) unreacted Hc concentration determined from the value of the absorption ratio A_{340}/A_{278} for oxy-Hc formed upon dilution of an aliquot into aerated buffer at pH 7; (■) NO_2^- concentration, determined by a modified Griess-Ilosvay assay; (□) half-met-Hc concentration, determined by quantitative EPR of Cu(II); (●) NO produced, determined by Ce(IV) reduction. The curves were generated by a least-squares fitting of the data using a general integrated rate law[14] for a second-order process, A + B → P. $k = 0.311 \pm 0.004$ M^{-1} h^{-1}. Reaction conditions: 2 mL of 1 mM deoxy-Hc in 50 mM propionic acid/Na propionate buffer, pH 5.5, containing 100 mM NaCl and 0.2 mM $NaNO_2$.

Hemocyanin (1 mM) was treated with limiting nitrite (0.2 mM) under an argon flow to maintain the protein in the deoxy form and at the same time remove the NO produced. Thus, formation of other oxides of nitrogen *in situ* was prevented. The gas phase was bubbled through a solution of Ce(IV) for quantitative analysis of NO. As the rate of the oxidation reaction was shown to be accelerated at low pH,[4,7] the solution was buffered at pH 5.5 to maximize the rate of conversion yet avoid protein denaturation during the experiment (∼24 h).[8] Chloride (100 mM) was added to the reaction mixture to prevent the binding of nitrite to the half-met product (see below).

In Figure 1, the time course of half-metHc formation is shown. At each of the indicated time points, an aliquot was withdrawn from the reaction mixture and analyzed for (a) the amount of unreacted Hc, determined in the aliquot diluted approximately 100-fold into aerated buffer and based on the ratio of intensities of the band at 278 nm[9] and that at 340 nm ($\epsilon \approx 20\,000$ M^{-1} cm^{-1}) characteristic of oxy-Hc,[10,11] where the 340 nm absorption is absent from half-met Hc; (b) the concentration of NO_2^-, determined spectrophotometrically in a modified Griess−Ilosvay assay;[6] (c) the amount of EPR active copper produced, quantitated by double integration of the Cu(II) EPR signal for the half-metHc derivative in its chloride form;[2,12]

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(8) Earlier work[7] suggested that HNO_2 was the oxidizing species in this reaction. Nitrous acid, however, is volatile and is also unstable in aqueous solution, disproportionating to NO and HNO_3. The yield of NO in our experiments, which was equal to the amount of NO_2^- consumed, rules out a significant interference from the spontaneous decomposition of HNO_2 at pH 5.5.
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and (d) the amount of NO generated, quantitated spectrophotometrically according to the change in absorbance due to Ce(IV) reduction.[13]

After 24 h incubation, the analysis showed that all the nitrite was consumed, 1 equiv of Hc was converted to half-met Hc, and 1 equiv of NO was produced. The time course could be curve-fit assuming a second-order process using a general integrated rate law.[14] The average kinetic constant derived from the least squares fitting is 0.311 ± 0.004 M^{-1} h^{-1}.[15]

The kinetics and material balance suggest a single redox process in the oxidation of deoxy Hc to half-met Hc.[16] Though the formation of metHc is unlikely with limiting nitrite, we ruled out the possibility that half-met Hc was formed from metHc and deoxy Hc in a separate experiment.[17] Furthermore, all the oxidizing equivalents transferred from NO_2^- to Cu(I) are accounted for as a single EPR active copper in half-met Hc. A noteworthy aspect of the proposed reaction scheme is its analogy to the single-electron reduction of nitrite catalyzed by the copper enzyme, nitrite reductase.[19]

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(12) X-band EPR spectra were recorded on a Varian E-112 spectrometer equipped with a Systron-Donner frequency counter and a Varian NMR gaussmeter. Data collection and double integration of the signal intensities were executed using a personal computer interfaced to the EPR spectrometer and EPR Data Acquisition System programs provided by P. D. Morse (University of Illinois).

(13) NO produced in the reaction was bubbled through a gas trapping column filled with glass helices and 25 mL of 6 mM ceric ammonium sulfate in 0.6 M H_2SO_4. The conversion to colorless Ce(III) was calculated based on absorbance measurements at 317 nm (ϵCe(IV) = 6000 M^{-1} cm^{-1}). The reduction of Ce(IV) and the apparatus for the transfer of NO from the reaction chamber to the cerium solution were standardized using known amounts of NO generated in an anaerobic reduction of nitrite by excess ascorbate. The stoichiometry of the reaction is expressed as

$$3Ce(IV) + NO + 2\ H_2O \leftrightarrows 3Ce(III) + NO_3^- + 4H^+$$

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(15) A similar reaction was performed with deoxy-Hc using 1 equiv of nitrite, in the absence of chloride. These conditions yielded only partial conversion of Hc to GHM-Hc due to the tight binding of nitrite to the half-met form. The bound nitrite is unavailable for the oxidation reaction. Chloride, shown to displace nitrite from GHM-Hc in an EPR titration (data not shown) was therefore added to the reaction mixture (100 mM) in the current experiment. A half-met chloride form of arthropod Hc has been reported[16] and the product of the reaction described here exhibited a similar EPR spectrum.

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(17) MetHc was produced using limiting H_2O_2 in the presence of excess deoxyHc.[18] No half-met Hc in its chloride form was detected by EPR after overnight incubation of the mixture (which contained a ratio of metHc to deoxyHc of 1:5) in the same buffer used for the reaction with nitrite (50 mM propionate, 100 mM NaCl, pH 5.5). This experiment demonstrates that the half-met derivative is not formed by single-electron exchange between the fully oxidized and fully reduced forms of Hc.

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