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KINETIC STUDIES OF THE COPPER NITRITE REDUCTASE FROM ACHROMOBACTER CYCLOCLASTES AND ITS INTERACTION WITH A BLUE COPPER PROTEIN

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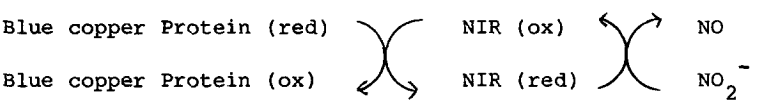
SUMMARY: Transient state, burst and steady state kinetics of reactions of the blue copper nitrite reductase (NIR) and blue copper protein from Achromobacter cycloclastes are investigated. The two copper-containing species are reacted with each other and where possible with dithionite, ascorbate and nitrite. Both copper proteins are fully reduced by dithionite with both $S_2O_4^{2-}$ and $SO_2^{\cdot-}$ species active. NIR is only partially reduced by ascorbate in an unusual biphasic reaction consistent with complete reduction of type-one copper followed by partial reduction of type-two copper. The rate of reduction of the type-one copper is accelerated using phenazine methosulfate as mediator. Nitrite can oxidize dithionite-reduced NIR but cannot reduce oxidized NIR. Rate constants were determined for all observed reactions.

Recent improvements in procedures for the isolation and purification of a blue copper protein from Achromobacter cycloclastes doubled its specific activity for reduction of the blue copper nitrite reductase (NIR) isolated from the same organism (1). The coupled reactions of the two copper proteins are:



In this communication we report the results of kinetic investigations of the above reaction scheme and of reductions using ascorbate and dithionite. The kinetics of ascorbate reduction of oxidized NIR were unusual in that under certain conditions two consecutive reductions were indicated by separable decreases in light absorbance.

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MATERIALS AND METHODS

Proteins: Blue copper protein and copper-containing NIR were isolated from Achromobacter cycloclastes IAM 1013 and purified by the method of Liu et al. (1). Protein quantities were determined by the method developed by Lowry et al. (2). Molecular weights measured by sedimentation equilibrium (3) were 12,000 for the blue copper protein and 69,000 for the NIR (1). The copper contents of these two proteins determined by plasma emission spectroscopy were one and three for the blue copper protein and NIR respectively. The NIR activity was measured by conventional Warburg manometry (4) and use of an oxides-of-nitrogen analyzer (1). The product of the NIR activity is predominantly nitric oxide (NO).

the reduced forms of the blue copper protein and NIR were prepared by adding a few crystals of dithionite to suspensions of the proteins, then removing the excess dithionite by passage through a Sephadex G-25 column (0.5x25 cm) under anaerobic conditions).

Stopped-flow Experiments: Reactions were studied at pH 7.0 in 0.2 M potassium phosphate buffer (30°C). Stopped-flow experiments were carried out in a Union Giken RA601 Rapid Reaction Analyzer. Solutions were made anaerobic by flushing with argon (zero gas, Canadian Liquid Air) purified by passage through an oxygen trap (Oxypurge N, Altech Associates). The argon flowed over the surface of the protein suspensions but was bubbled through all other solutions. Gas-tight Hamilton syringes were employed for all manipulation of fluids. Sodium dithionite stock solutions contained a weighed amount of the reductant added to 5 mL anaerobic buffer, pH 7.0. The concentrations of the dithionite solutions were determined by titration with potassium ferricyanide (5). The kinetics of all reactions of the blue copper protein and NIR were monitored by following changes of light absorption at 595 nm and 458 nm, respectively.

RESULTS

The kinetics of reduction of NIR by ascorbate were studied under pseudo-first order conditions both in the presence and absence of phenazine methosulfate (PMS). Unusual biphasic kinetics were observed (Fig. 1). Second order rate constants for

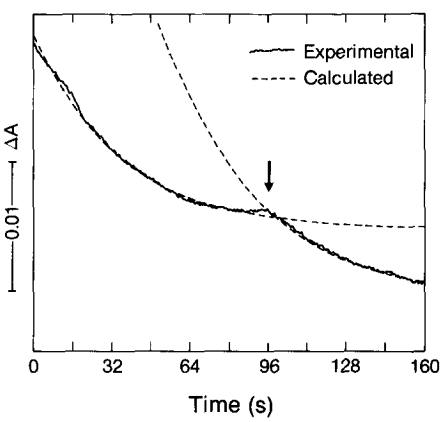


Figure 1. Absorbance change at 458 nm with time for the reaction of NIR with ascorbate as observed on the stopped-flow apparatus. Final concentrations were 3.42 μM NIR and 30.0 mM ascorbate. The reaction was studied at pH 7.0 and 30°C. The arrow indicates the beginning of the second step.

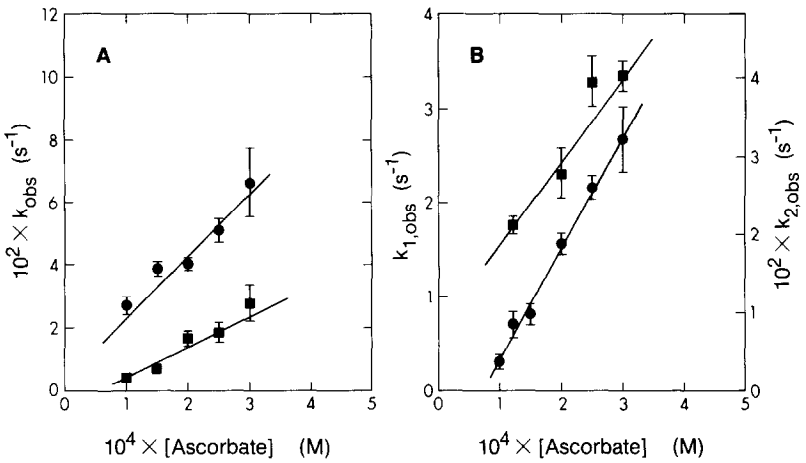


Figure 2. Plots of k_{obs} versus ascorbate concentration for the reduction of NIR with ascorbate in the absence (A) and in the presence of PMS (B). (●) k_{obs} for the first process and (■) k_{obs} for the second process. The slopes yield the second order rate constants. Note the different ordinate scales in (B). Left scale for the first process, right for the second.

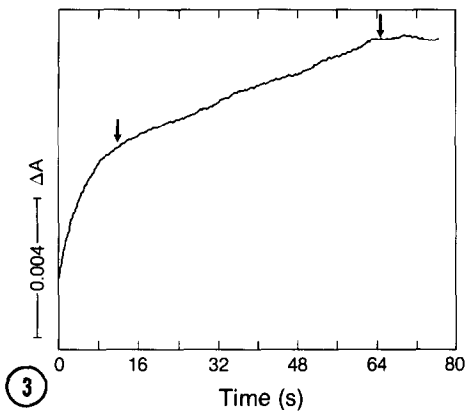
both processes were determined from the slopes of the plots of the observed pseudo-first order rate constants versus the ascorbate concentrations (Fig. 2). The rate constants for the two steps of the reaction are (2.2±0.3) $M^{-1}s^{-1}$ and (1.1±0.1) $M^{-1}s^{-1}$ in the absence of PMS, and (1.2±0.1) x 10^2 $M^{-1}s^{-1}$ and (1.1±0.1) $M^{-1}s^{-1}$ in the presence of PMS.

Assays of rates of dithionite reduction of both NIR and blue copper protein in reaction mixtures supplemented with a large excess of dithionite yielded rate constants, k_{obs}, first order with respect to time, versus $[S_2O_4^{2-}]$ or versus $[S_2O_4^{2-}]^{1/2}$, that were not linear (data not shown). For dithionite reduction of both copper proteins the data are fitted by the equation

$$k_{obs} = a[S_2O_4^{2-}] + b[S_2O_4^{2-}]^{1/2}$$

where a and b are, respectively, (2.8±0.9) x 10^2 $M^{-1}s^{-1}$ and (1.2±0.1) x 10^2 $M^{-1/2}s^{-1}$ in the case of NIR, and (4.1±0.8) x 10^4 $M^{-1}s^{-1}$ and (6.9±1.8) x 10^2 $M^{-1/2}s^{-1}$ for the blue copper protein.

The oxidation of dithionite-reduced blue copper protein by nitrite was studied, with NIR serving as catalyst. In one series of experiments the NIR concentration was held constant and the concentration of nitrite varied; in another the converse applied. At constant NIR concentration (with mole/mole ratio



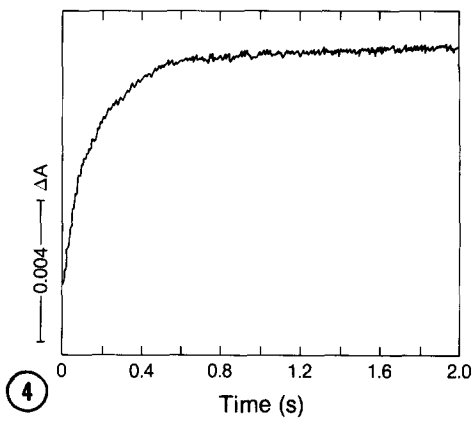


Figure 3. Absorbance changes with time for the reaction of dithionite-reduced blue copper protein (9.4 μM) with 0.1 M nitrite in the presence of 3.2 μM oxidized nitrite reductase as catalyst. The trace was recorded on the stopped-flow apparatus at 595 nm, pH 7.0 and 30°C. The arrows indicate the limits of the zero order process.

Figure 4. Absorbance changes with time for the reaction of dithionite-reduced blue copper protein (9.4 μM) with 0.1 M nitrite in the presence of 9.4 μM nitrite reductase as catalyst. Other conditions as described in the Figure 3 caption.

of NIR to reduced blue copper protein, 1:3) a first order process was followed by a zero order process (Fig. 3). When the ratio of NIR to reduced blue copper protein was 1:1, only one process was observed (Fig. 4). The rate constant k_{obs} for the first step was independent of nitrite concentration over the range (1-20) x 10^{-4} M with a mean value of (1.6±0.2) s^{-1}. The zero order rate constants were linearly dependent upon nitrite concentration. The slope of the plot of $k_{z.o.}$ *versus* nitrite concentration gave a value of (9.0±1.0) x 10^{-2} s^{-1} for the second process. At constant nitrite concentration the k_{obs} values were linearly dependent on NIR concentration. The second order rate constant, determined from the slope of the plot of k_{obs} *versus* NIR concentration is (9.7±0.7) x 10^5 $M^{-1}s^{-1}$ (data not shown).

When nitrite was mixed with oxidized NIR, no changes in absorbance at 458 nm occurred. On the other hand dithionite-reduced NIR is oxidized by nitrite in a pseudo-first order process. The slope of a plot of k_{obs} *versus* nitrite concentration gave the second order rate constant for NIR oxidation of (2.4±0.4) $M^{-1}s^{-1}$ (data not shown).

DISCUSSION

By analogy with other systems, it was hypothesized that a c-type cytochrome was the electron donor for the copper-containing NIR (4,9,10). However a small-molecular-weight blue copper protein was found in both A. cycloclastes (1,4) and Alcaligenes faecalis strain S-6 (7,8). Several subsequent observations suggested that the small-molecular-weight blue copper protein could serve as electron donor for the copper-containing NIR (1,8), which can in turn donate an electron to nitrite to reduce it to the predominant product, NO (1,4).

Both plasma emission spectroscopy (1) and epr studies (Moura et al., unpublished results) are consistent with the characteristic occurrence of one atom of copper per mole of blue copper protein and three copper atoms per mole of copper-containing NIR.

Ascorbate can fully reduce the blue copper protein but only partially reduce the NIR. The latter protein can be fully reduced by dithionite. The observed two-step kinetics of reduction of oxidized NIR were unexpected. Ascorbate may be reducing type-one copper of NIR completely and starting to reduce the type-two copper. Not surprisingly, PMS can act as an electron carrier. The results indicate that the rate constant for the first step is increased by about 50-fold in the presence of PMS as mediator, but the second step remains unaffected by the mediator (Fig. 2). The kinetics of reduction of both NIR and blue copper protein by dithionite are consistent with a mechanism in which both $S_2O_4^{2-}$ and SO_2^- species are kinetically involved reducing species. A similar kinetic behaviour for dithionite reduction was previously reported for ferricytochrome c and plastocyanin (5). The rate constants for dithionite reduction of blue copper protein are higher than for NIR.

When dithionite-reduced blue copper is mixed with NIR as catalyst the reaction occurs in two steps: an initial burst followed by steady-state kinetics (Fig. 3). The rate constant for the burst phase is independent of nitrite concentration. However, when the concentration of blue copper protein to NIR is 1:1 only the burst kinetics were observed (Fig. 4). The observed steady-state kinetics are nitrite concentration dependent. The first order rate constant,

obtained from the slope of the plot of observed zero order rate constant versus nitrite concentration, appeared to be about 20-fold less than that for the burst phase.

In the presence of nitrite as catalyst, the oxidation of dithionite-reduced blue copper protein with NIR follows second-order kinetics with a rate constant of $(9.7\pm0.7) \times 10^5\ M^{-1}s^{-1}$.

The results of studies involving nitrite dependence or independence also indicate that nitrite can only oxidize dithionite-reduced NIR and cannot reduce oxidized NIR. The rate constant for the nitrite oxidation of NIR is $(2.4\pm0.4)\ M^{-1}s^{-1}$.

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