# THE NITRIC OXIDE COMPOUNDS OF *PSEUDOMONAS AERUGINOSA* NITRITE REDUCTASE AND THEIR PROBABLE PARTICIPATION IN THE NITRITE REDUCTION

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## 1. Introduction

Differing from nitric oxide compounds of ordinary hemoproteins such as Hb, Mb and cytochrome *c*, those of *Pseudomonas aeruginosa* nitrite reductase, which possesses two different hemes *d* and *c* in a molecule, deserve special attention, because NO is an immediate product of this enzyme-catalyzed reduction of nitrite [1] and the NO compounds may have physiological significance in the reaction. Under anaerobic conditions NO was found to alter the absorption spectrum due to heme *d* of the either ascorbate- or dithionite-reduced *Ps.* enzyme [2], and participation of the heme *d* in the enzymic reduction of nitrite was suggested by Yamanaka and Okunuki [2] and Kijimoto [3]. However, participation of the heme *c* moiety in either the enzymic reaction or the NO compound formation is open to further investigations. In the present report, we will show that *Ps.* nitrite reductase in both oxidized and reduced states forms with NO two distinct compounds at both hemes, and that these compounds, in addition to the oxidized and reduced enzymes, are formed during the turnover of this enzyme as functional intermediates.

## 2. Materials and methods

*Pseudomonas aeruginosa* nitrite reductase was extracted from the cells grown anaerobically in the presence of nitrate, according to the method of Horio et al. [4], and purified by chromatography on DEAE- and CM-celluloses. Finally it was crystallized from 10 mM Tris–HCl buffer (pH 7.5) [5]. The concentration of the reductase was determined by using a millimolar extinction coefficient of 30.2 at 549 nm for the reduced pigment [4].

The absorption spectra were recorded on a Cary Model 16 spectrophotometer equipped with the automatic recording accessory. All the spectral measurements were carried out by using Thunberg type cuvettes of 1 cm light path, and the anaerobiosis was attained usually by repeated evacuations and washings with pure Argon gas at room temperature. When necessary, NO gas was finally introduced to a cuvette.

NO gas was obtained from Takachiho Chemical and Technological Co. Ltd., and used without further purification.

## 3. Results and discussion

### 3.1 *NO compound of Ps. nitrite reductase*

The oxidized enzyme in a NO-saturated medium formed NO compounds at both hemes at pH's below neutrality. At pH 5.7 where the enzyme activity was usually assayed, a stable spectrum appeared having a symmetrical peak at 634 nm and distinct twin peaks at 560 and 529 nm (fig.1). The shape and peak positions of the latter closely resembled those of mammalian ferricytochrome *c*-NO (6–8). This compound formation was pH-dependent and complete at pH 3.9. Interestingly, the pH 5.7-spectrum was synthesized from 0.7 of the pH 3.9-spectrum and 0.3 of the oxidized one at neutral pH throughout the wavelength region between 700 and 500 nm. These values as well as the concentration of NO in the solution, calculated from its known solubility in water, tentatively gave a dissociation constant of 1 mM for the compounds at both hemes. However, such a uniform behavior

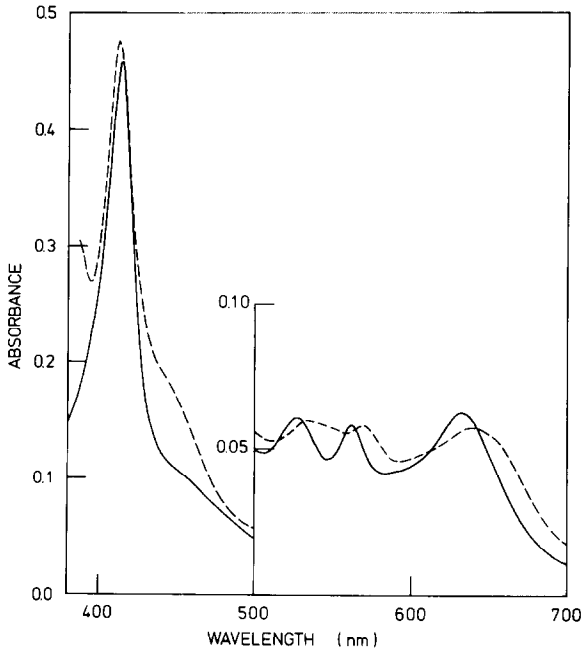



Fig.1. Absorption spectra of the NO compounds of *Pseudomonas* nitrite reductase. To the main room of a Thunberg type cuvette were added 2.5 ml of 0.2 M sodium phosphate buffer (pH 6) and 0.025 ml of 180 μM *Pseudomonas* enzyme. After deaeration the oxidized-NO compound was formed by introducing anaerobically NO gas into the cuvette (——), and then it was reduced by addition of 0.02 ml of 1 mM PMS and 0.01 ml of 50 mM NADH from the side arm (– – –). The final pH of the reaction mixture was 5.72.

of the hemes *d* and *c* was fortuitous, since at pH 5.6 the heme *d* exceeded slightly the heme *c* in the extent of the compound formation. Above pH 6.1, the oxidized enzyme was reduced partially, probably according to a mechanism proposed by Ehrenberg and Szczepkowski [6].

When the oxidized-NO compound was reduced with NADH and a catalytic amount of PMS, a trough between the twin peaks in the heme *c* region became shallower and the peaks shifted to 568 and 534 nm, respectively, resembling mammalian ferrocytochrome *c*-NO in shape and peak position. In the heme *d* region the 634-nm peak decreased in intensity and shifted to 640 nm accompanying a new shoulder at around 655 nm (fig.1). This spectral change was induced by ascorbate, too. The reduced-NO compound was also obtained by exposing either ascorbate- or NADH + PMS-reduced enzyme to NO gas. Keilin et al. [7,8] found and named mammalian ferricytochrome *c*-NO and ferrocytochrome *c*-NO as Compounds I and II, respectively. Therefore, our oxidized- and reduced-NO compounds will be referred to in the same way.

### 3.2 *Steady state spectra.*

The reaction was initiated by the anaerobic addition of nitrite to a solution of the enzyme which had been reduced and supplemented with an excess of NADH + PMS, and the absorbance decrease was followed at 340 nm (fig.2). The absorption spectra of the reaction mixture recorded on the same time scale apparently indicate the immediate establishment of the steady state. The shape of this spectrum, however, changed depending on the relative concentrations of NADH and nitrite initially added to the reaction mixture (fig.3, A and B). In none of these two cases the

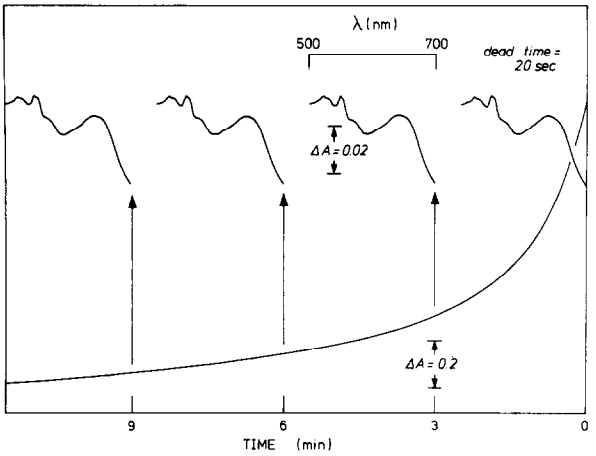



Fig.2. Utilization of NADH by *Pseudomonas* nitrite reductase with nitrite as an electron donor. To the main room of a Thunberg cuvette were added 2.7 ml of 0.2 mM sodium phosphate buffer (pH 7), 0.02 ml of 1 mM PMS and 0.03 ml of *Ps.* reductase. After the enzyme had been reduced by 0.03 ml of 33 mM NADH supplied through a microsyringe 0.25 ml of 0.5 M sodium phosphate buffer (pH 6) in the side arm was added to adjust the pH to 6.08. The reaction was initiated by a further addition of 0.03 ml of 40 mM sodium nitrite through another microsyringe. The final concentration of the *Ps.* enzyme was 2 μM. In a separate experiment, the reaction mixture of the same composition was prepared and processed in the same way, except that the absorption spectra were recorded repeatedly in accordance with the recorded absorbance change.

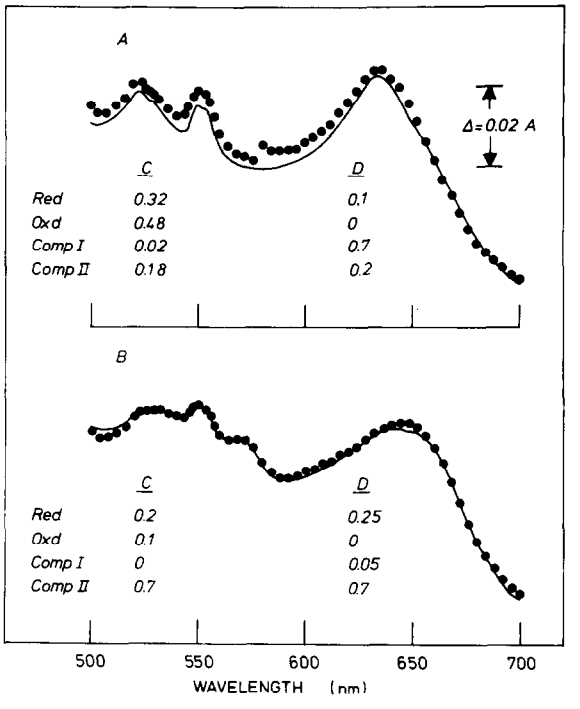
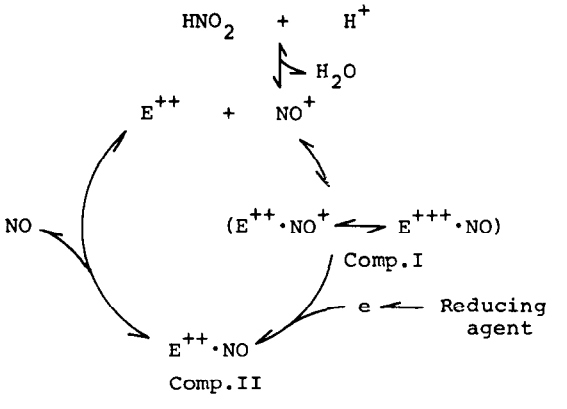



Fig.3. The steady state spectra of *Pseudomonas* nitrite reductase. The steady state spectra were recorded as described in the legend for fig.2. The main room of a Thunberg cuvette contained 2.6 ml of 0.2 mM sodium phosphate buffer (pH 7), 0.02 ml of 0.1 mM PMS and 0.03 ml of *Ps.* enzyme, the side arm containing 0.25 ml of 0.5 M sodium phosphate buffer (pH 6) to bring the final pH to 6.07. The further additions made through microsyringes were (A) 0.015 ml of 10 mM NADH and 0.03 ml of 10 mM sodium nitrite, and (B) 0.03 ml each of the two solutions. The final concentration of the *Ps.* enzyme was 2.4 μM. The lines are the recorded spectra and the small filled circles are the calculated values. For the computation the absorption spectra of Comp. I and II at pH 5.72 were used tentatively.

spectrum was composed of the spectra of only the oxidized and reduced enzymes. Indeed, we could synthesize by trial and error the steady state spectra using the spectra of Comp. I and II, in addition to the above two. The computation was carried out separately for the heme *d* region between 700 and 580 nm and for the heme *c* between 580 and 500 nm. The agreement between the synthesized and experimental spectra was quite satisfactory if we consider the spectral overlapping of one of the hemes on the other. The estimated relative concentrations of each component in the steady state are summarized in the insert for fig.3, and it is apparent that the hemes *d* and *c* behave either differently (A) or alike (B). Also, the 570 nm peak preciously ascribed to the reaction of NO with the free ascorbate radical [2] was concluded to be due to Comp. II. As the reducing power was increased (A → B), different behaviors of the two hemes towards the reactants disappeared and the contribution of Comp.II became prominent. Since either Comp.I or the oxidized form was prominent with a weak reducing power, this change would correspond to the enhanced reduction of Comp.I to II in the functioning states.

Now, it is worth speculating from what NO compounds originate during the reaction. It is known that nitrous acid dissociates into $NO^+$ and water at acidic pH's [9]. From kinetic analyses of the reaction of mammalian ferrocytochrome *c* with nitrite, we could reveal that the active species of nitrite was $NO^+$ at lower pH's (see also [6]), whereas it was $N_2O_3$, an oxidant, at pH's near neutrality [10]. Whether $NO^+$ or $N_2O_3$ is acting is determined not only by the pH of the reaction mixtures but also by the chemical nature of the prosthetic groups and chromophoric environments in the hemoproteins. Thus, at higher pH's the reduced *Ps.* enzyme would be more reactive to $NO^+$ than mammalian ferrocytochrome *c*, and the abundance of the oxidized form at the heme *c* moiety (fig.3, A) would indicate that it is more vulnerable to the attack of $N_2O_3$ than the heme *d*.

The Comp.I formation from the oxidized enzyme




Scheme 1. A proposed reaction mechanism of the nitrite reduction.

and NO under turnover conditions is less possible than that from the reduced enzyme and $NO^+$, since the former is governed by the high dissociation constant of 1 mM. Therefore, the reaction sequence that the reduced enzyme reacts with nitrite through $NO^+$ forming Comp.I, and that it in turn is reduced to Comp. II receiving electrons enabled us to present the following scheme for the enzymic reduction of nitrite. This scheme does not specify if one or both of the two hemes are involved, although at least in the presence of a strong reducing system both hemes must be functioning.

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