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Highly efficient control of iron-containing nitrile hydratases by stoichiometric amounts of nitric oxide and light

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Abstract The reaction of two iron-containing nitrile hydratases (NHase) with NO has been studied: NHase from *Rhodococcus* sp. R312, which is probably similar to the photosensitive N771 NHase, and the new NHase from *Comamonas testosteroni* NI1 whose aminoacid sequence is quite different from those of BR312 and N771 NHases. Both enzymes are equally inactivated after addition of stoichiometric amounts of NO added as an anaerobic solution or produced in situ under physiological conditions by a rat brain NO-synthase. Both enzymes are reactivated by photoirradiation, and two cycles of NO inactivation/photoactivation can be performed without significant loss of activity. Both iron-containing NHases have a high affinity for NO, similar to that of methemoglobin.

Key words: *Rhodococcus* sp. R312 or BR312; *Comamonas testosteroni* NI1; Charge transfer band; Fe–NO bond

1. Introduction

Nitrile hydratases (NHases) are bacterial enzymes that catalyse the specific hydration of nitriles into the corresponding amides [1]. Iron-containing NHases from *Rhodococcus* sp. R312 (originally *Brevibacterium* sp. R312 or BR312) [2,3], *Rhodococcus* sp. N774 [4], *Rhodococcus* sp. N771 [5] and *Pseudomonas* sp. B23 [6] all involve in their active site a characteristic low-spin non-heme iron(III) centre. The NHase from *Rhodococcus* sp. N771 isolated from cells grown in the dark exists under an inactive form which can be converted into a catalytically active form after light irradiation [5]. The N771 NHase inactive form exhibits two bands in UV-vis spectroscopy at 280 and 370 nm [7], and appears to involve two different iron oxidation states, iron(II) and iron(III), on the basis of Mössbauer, ESR spectroscopy and magnetic susceptibility measurements [8]. The iron(II) centre would be oxidized to the iron(III) state during the photoactivation process, and the photoactivated enzyme is characterized by two peaks at 280 and 710 nm in UV-vis spectroscopy [7]. Recently, a FTIR study has indicated that a NO molecule is bound to the iron centre of inactive N771 NHase, and it has been proposed that NO could be involved in the photoactivation process [9]. This NO molecule and the iron centre appear to be located in the α subunit of N771 NHase [10].

As an increasing number of proteins appear to be regulated (or inactivated) by NO [11–14], it seemed interesting to study the influence of NO, either externally added as a solution or produced in situ by a NO-synthase, on the active form of iron-containing nitrile hydratases. Two such enzymes have been studied: NHase from *Rhodococcus* sp. R312, an $\alpha^2\beta^2$ tetramer of 95 kDa containing two iron atoms per protein [3], which is probably similar to N771 NHase because of their identical amino-acid sequences [15,16], and NHase from *Comamonas testosteroni* NI1, that specifically catalyzes the hydration of 5-cyanovaleric acid in adipamic acid [17]. The latter enzyme is an $\alpha^2\beta^2$ tetramer of 95 kDa, whose amino-acid sequence is quite different from those of BR312 and N771 NHases.

This paper reports that both active NI1 and BR312 NHases are inactivated after addition of **stoichiometric** amounts of NO, either added as a solution under anaerobic conditions or produced in situ by a NO-synthase associated to the NHase and working under aerobic physiological conditions. It also shows that these NO-inactivated NHases completely recover their activity and initial UV-vis spectrum upon photoirradiation. These results indicate that the regulation by NO and light, that was first found for N771 NHase, could be general for iron-containing NHases, and points to the high efficiency of NHase inactivation which occurs with stoichiometric amounts of NO even under physiological aerobic conditions.

2. Material and methods

Unless otherwise specified, all experiments were performed in 100 mM Hepes-KOH buffer, pH 7.2, referred as Hepes buffer. Absolute and differential UV-Vis spectra were recorded in 1 cm quartz cuvettes with a UVIKON 820 spectrophotometer thermostated at 10°C.

2.1. Enzyme purification

BR312 and NI1 NHases were purified and stored in the presence of butyric acid (40 mM) as previously described in the literature [2] and according to the procedure described in an industrial patent [17], respectively. Purified rat brain NO synthase (rbNOS) was kindly provided by M.A. Sari et al. [18]. Just before use, rbNOS was buffer-exchanged through a sephadex G-50 column with 50 mM Hepes buffer pH 7.4 containing 5 mM dithiotreitol (DTT).

2.2. Preparation of NO solution and titration

NO gas was supplied from Aldrich. Saturated aqueous solutions of NO were prepared by bubbling NO gas during 10 min into a septum-capped tube containing Hepes buffer carefully purged with argon prior to NO saturation. NO solutions were used without further treatment to remove higher oxides. Freshly prepared NO solutions were titrated according to the oxyhemoglobin (HbO_2) method described by Murphy et al. [19]. HbO_2 concentration was determined by the 415 nm absorbance ($\varepsilon = 131\ mM^{-1}\cdot cm^{-1}$). NO solutions were titrated between 2 and 2.1 mM. Diluted NO solutions were obtained by a 25-fold dilution of the 2 mM NO stock solution in deaerated Hepes

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Abbreviations: NHase, nitrile hydratase; EPR, electron paramagnetic resonance; FTIR, Fourier transform infrared; rbNOS, rat brain nitric oxide synthase; $HbFe^{III}$, methemoglobin; HbO_2, oxyhemoglobin

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buffer. Diluted NO solutions were also titrated using the HbO_2 method.

2.3. Inactivation of NHase by NO solution

12 µl of NHase (14 mg/ml) were added to 200 µl of Hepes buffer leading to a final concentration of 8 µM NHase. The NHase solution was then divided into the sample and reference deaerated cuvettes and a baseline was recorded between 880 nm and 280 nm. Successive volumes of 0.2–0.4 µl of NO were added every 3 min into the sample cuvette and a difference spectrum was recorded after each addition.

2.4. Photoactivation of NHase

The inactive nitrosylated NHase, maintained in an ice bath, was exposed to the white light of a Bioblock 150 W lamp in aerobic conditions. Photoactivation occurred within 15–45 min and was monitored by UV-Vis spectroscopy.

2.5. NHase activity assay

NHase activity was assayed by following the hydration of acrylonitrile for BR312 and 5-cyanovaleric acid for NI1. To a glass test tube containing 1 ml buffer solution of the substrate (17 mM acrylonitrile or 2.5 mM cyanovaleric acid for BR312 and NI1, respectively) were added 6 µl of 0.3 µM NHase in Hepes buffer (coming from a 30-fold dilution of the sample cuvette). The tubes were incubated at 28°C for 10 min and the reaction stopped by addition of 1 ml of 0.1 N HCl. Acrylamide or adipamic acid formation was monitored at 208 nm using a Gilson HPLC system equipped with a Hypersyl C18 column eluted with a mixture of acetonitrile/5 mM phosphate buffer (5/95) at a flow rate of 1 ml/min.

2.6. Competition experiments

In all experiments, NO was added into the sample cuvette by 0.2–0.4 µl aliquots of a 2 mM NO stock solution. The respective concentrations of NI1 NHase and $HbFe^{III}$ were 8 µM and 4 µM. The relative contents of the sample and reference cuvettes were as follows:

2.6.1. $HbFe^{III}$+NO/Hepes. The absolute spectrum of $HbFe^{III}$ was recorded versus Hepes buffer and the nitrosylation of $HbFe^{III}$ was characterized by the decrease of the absorbance at 403 nm.

2.6.2. $HbFe^{III}$+NHase+NO/NHase or NHase+$HbFe^{III}$. In order to follow the nitrosylation of $HbFe^{III}$ and NHase during the competition experiments, spectral changes were monitored at 403 nm versus NHase and at 680 nm versus a mixture of NHase+$HbFe^{III}$.

2.7. NHase and NO synthase in vitro coupling

All the following procedures were carried out in 300 µL of 50 mM Hepes buffer, pH 7.4, at 28°C in aerobic conditions, with a UVIKON 942 spectrophotometer. All solutions contained 1 mM $CaCl_2$, 4 µM FAD, 4 µM FMN, 5 µM BH_4, 0.2 mM DTT and 100 units/ml calmoduline as rbNOS cofactors and 100 µM L-arginine and 300 µM NADPH as NOS substrates.

2.7.1. rbNOS + HbO_2. The NOS activity was determined by the oxyhemoglobin assay. Difference spectra of HbO_2 (20 µM) was monitored by UV-vis spectroscopy for 12 min following the addition of 8 nM rbNOS in the presence of all the cofactors and the substrates at the above mentioned concentrations. The initial rate of NO production measured over 2 min was 0.55 µM NO per min.

2.7.2. rbNOS + NHase. Difference spectra of BR312 or NI1 NHases (14 µM) was monotored by UV-vis spectroscopy for 11 min after addition of 44 nM rbNOS. Absolute spectra of sample and reference cuvettes were then recorded versus Hepes buffer.

3. Results

3.1. Inactivation of BR312 and NI1 NHases by NO

Two active NHases have been compared in this study, the well-known BR312 NHase and the new NI1 NHase from *Comamonas testosteroni* [17]. Preliminary ESR and atomic absorption spectroscopy studies performed on NI1 NHase, purified in the presence of 40 mM butyric acid, showed that the $\alpha^2\beta^2$ tetramer contains two low spin iron(III) atoms, like BR312 NHase (data not shown). Its electronic spectrum is very similar to that reported for BR312 NHase, with a band at 280 nm, a shoulder around 400 nm and a clear less intense

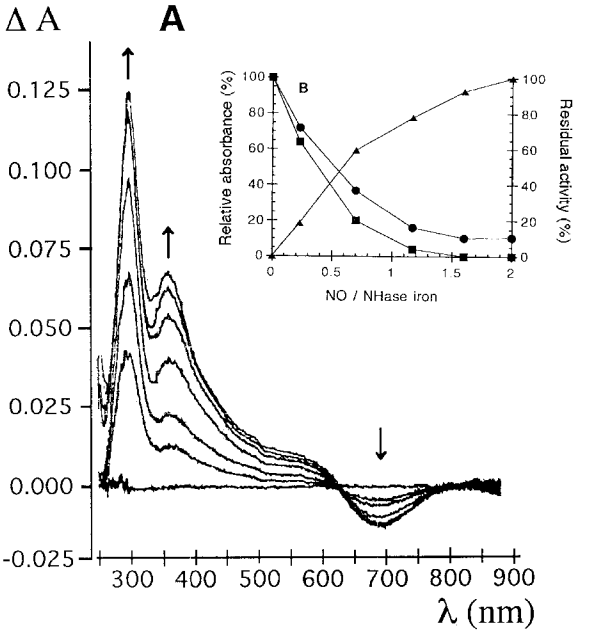

Fig. 1. Effects of NO on the UV-visible spectrum and NI1 NHase activity. A: difference spectra of 8 µM NI1 NHase in 100 mM Hepes buffer pH 7.2 after addition of 0.23, 0.46, 0.7, 1.17, 1.6 and 2 equivalents of NO per NHase iron. Arrows indicate the reaction progress. B: changes in absorbance at 680 nm (■) and 365 nm (▲) deduced from A, and in the corresponding activity of NI1 NHase towards hydration of 5-cyanovaleric acid (●). Typical experiment representative of three other ones within ±15%.

band at 680 nm, that has been assigned to a sulfur-to-iron charge transfer band as in the case of BR312 NHase [3]. NI1 NHase is also stabilized by the presence of 40 mM butyric acid that acts as a competitive inhibitor of 5-cyanovaleric acid hydration, with a K_i value of 2.4 mM (data not shown). This value is similar to that reported for the competitive inhibition of BR312 NHase by butyric acid [20,21].

Addition of increasing amounts of an anaerobic solution of NO to 8 µM NI1 NHase led to the progressive loss of the charge transfer band at 680 nm and to the concomitant appearance of a band at 365 nm. The difference spectra corresponding to this phenomenon exhibited an isosbestic point at 620 nm and are shown in Fig. 1A. Active BR312 NHase exhibited a very similar behaviour towards NO (Fig. 1B). Its final UV-vis spectrum characterized by two bands at 280 and 370 nm is almost identical to that previously reported for inactive N771 NHase [7]. The progressive changes observed in UV-vis spectroscopy upon addition of NO to NI1 or BR312 NHase are directly correlated to the loss of their enzymatic activity (hydration of 5-cyanovaleric acid for NI1 NHase, Fig. 1B, and hydration of acrylonitrile for BR312 NHase, Fig. 2B). This correlation clearly shows that BR312 NHase is completely inactivated and transformed into the 370 nm-absorbing form after addition of 1.7 ± 0.2 equivalents of NO per iron atom. Similarly, 2 ± 0.2 equivalents of NO per iron atom are required for maximal inactivation of NI1 NHase and its transformation into a 365 nm-absorbing form.

The effects on NHases of other nitrosylating agents, $NOBF_4$ or S-nitroso-*N*-acetyl penicillamine (SNAP), that may act as either NO^+or NO donors respectively, [22] have been similarly studied. Whereas less than two equivalents of NO led to a complete disappearance of the 710 nm band of 8

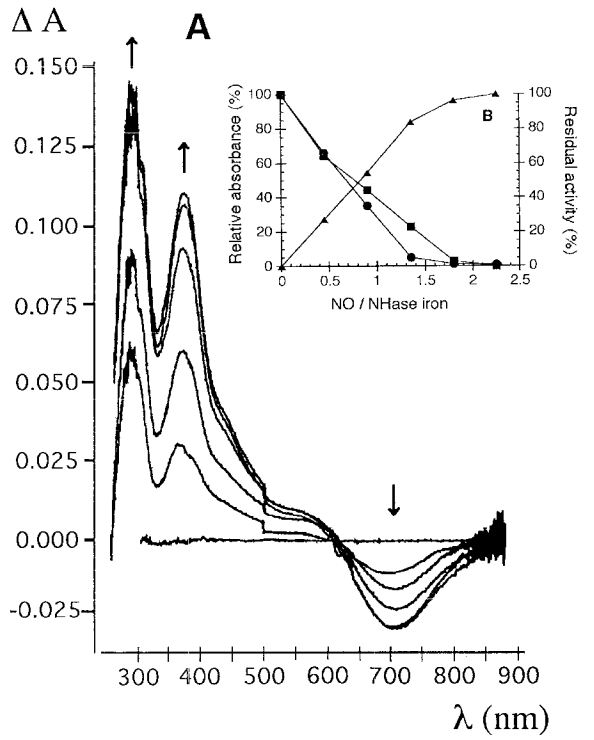

Fig. 2. Effects of NO on the UV-visible spectrum and BR312 NHase activity. A: difference spectra of 8 μM BR312 NHase after addition of 0.45, 0.9, 1.35, 1.8 and 2.25 equivalents of NO per NHase iron. Conditions identical with those of Fig. 1A. B: changes in absorbance at 710 nm (■) and 370 nm (▲) deduced from A, and in the corresponding activity of BR312 NHase towards hydration of acrylonitrile (●). Typical experiment representative of two other ones within ±10%.

μM BR312 NHase, 25 equivalents of $NOBF_4$ were required for the same effect, and no spectral change was observed after addition of up to 30 equivalents of SNAP per NHase iron (data not shown). The latter result is probably due, in our conditions, to the lack of any photochemical or redox activation of SNAP that is required for NO formation [23].

3.2. Competitive reactions of NO with NHase and methemoglobin ($HbFe^{III}$)

The aforementioned inactivation of NHases with nearly stoichiometric amounts of NO suggests a high affinity of NO towards the NHase iron centre. In order to have a better idea of this affinity, we have performed competitive experiments between NHases and methemoglobin ($HbFe^{III}$). The overall reaction of $HbFe^{III}$ with NO is a reductive nitrosylation of the iron active site, which occurs in two steps (Eqn. 1) and finally leads to the formation of HbFe(II)-NO [24].

$$\begin{aligned} &\text{HbFeIII} + \text{NO} \rightleftharpoons \text{HbFeIII–NO} \quad \rightarrow \text{HbFeII–NO} + \\ &\text{HbFeII} + \text{NO} \rightarrow \text{HbFeII–NO} \end{aligned} \qquad (1)$$

The decrease of absorption at 403 nm, that is characteristic of $HbFe^{III}$ nitrosylation, follows a linear variation with the number of NO equivalents added, whether NI1 NHase is present or not in the incubation mixtures (Fig. 3A). The ratio of the slopes observed in the presence or in the absence of NI1 NHase allowed us to show that about 50% of NO reacts with $HbFe^{III}$ in incubations containing a 1:2 mixture of $HbFe^{III}$ and NHase (1:1 ratio in iron content). Similar competitive experiments using NO and $HbFe^{III}$-NHase mixtures and monitoring the decay of the 680 nm charge transfer band of NI1 NHase (Fig. 3B) led to the same conclusion that NO reacts equally well with NHase Fe^{III} and $HbFe^{III}$.

3.3. Photoactivation of NO-inactivated NI1 and BR312 NHases

Simple irradiation of NI1 and BR312 NHases previously inactivated by NO by the white light of a 150 W lamp under aerobic conditions at 4°C completely restores the UV-vis spectrum of the active enzymes (disappearance of the bands at 365 or 370 nm and appeareance of the bands at 680 and 710 nm, respectively) as well as their initial enzymatic activity (data not shown). In order to investigate a possible in vivo regulation of NHase by NO and light, we have performed several NO-inactivation/light-induced reactivation cycles on the same sample. NI1 NHase may undergo two such cycles, involving first an inactivation step after addition of four NO equivalents per iron and then a photoactivation step, with a complete recovery of its initial enzymatic activity (Fig. 4). After 3

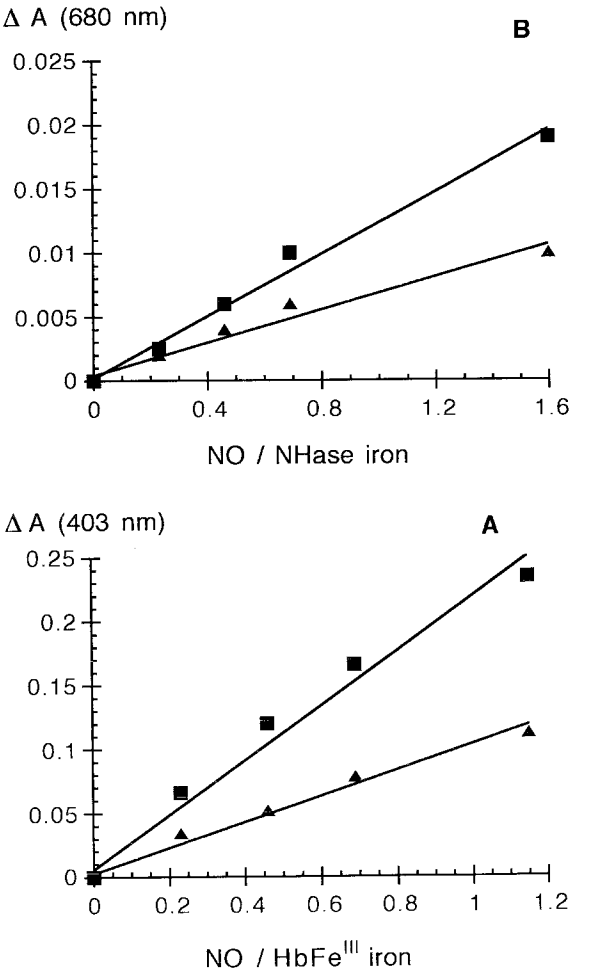

Fig. 3. Reaction of $HbFe^{III}$ with NO with or without NI1 NHase. A: plot of the decrease of the absorbance at 403 nm of $HbFe^{III}$ (4 μM in 100 mM Hepes buffer pH 7.2) as a function of increasing equivalent numbers of NO, in the presence (▲) or absence (■) of 8 μM NI1 NHase. B: plot of the decrease of the absorbance at 680 nm of NI1 NHase (8 μM in 100 mM Hepes buffer pH 7.2) as a function of increasing equivalent numbers of NO, in the presence (▲) or absence (■) of 4 μM $HbFe^{III}$. Typical experiment representative of two other ones within ±10%.

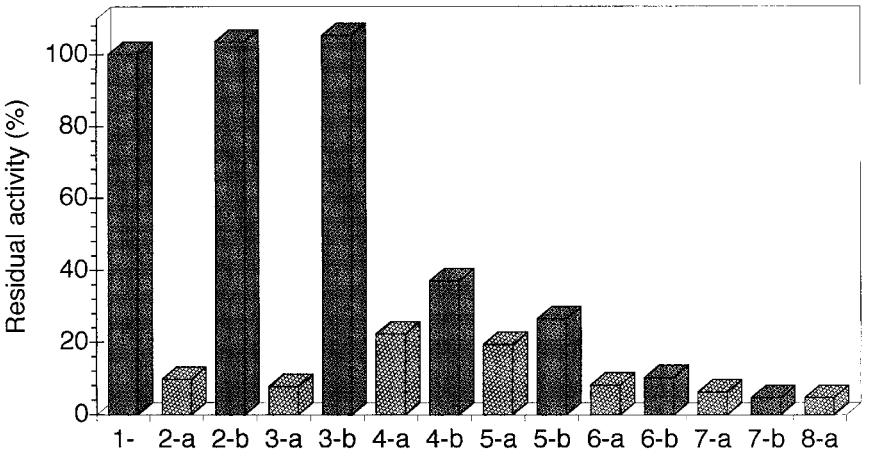

Fig. 4. Evolution of the NI1 NHase activity towards hydration of 5-cyanovaleric acid after successive cycles of inactivation by NO and photoactivation. (1) Initial NHase activity (100% = 95 ± 15 μmol·min^{-1} ·(mg protein)$^{-1}$); (2)–(8) Enzymatic activity of successive cycles of: (a) addition of four equivalents of NO per NHase iron to 8 μM NHase in 100 mM Hepes buffer pH 7.4; (b) photoactivation of the enzyme with a white light during 30 min at 4°C. Typical experiment representative of two other ones within ± 10%.

cycles, 40% of the activity is restored, then the enzymatic activity progressively decreases after further cycles. This irreversible inactivation of the enzyme should be due to the secondary reactions promoted by NO in excess. BR312 NHase was found to undergo similar NO-inactivation/photoactivation cycles but with a greater tendency to be irreversibly inactivated at least partly after three cycles (data not shown).

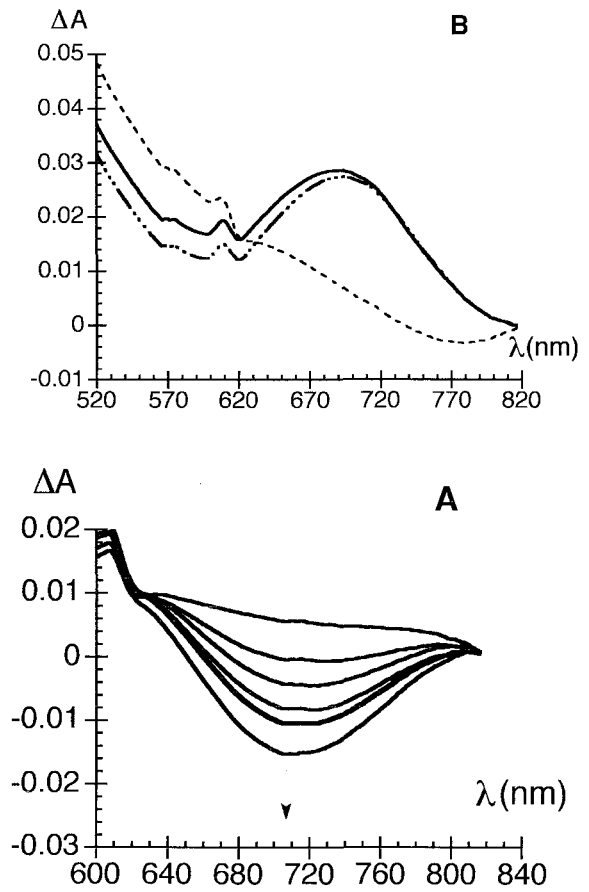

Fig. 5. Inactivation of NI1 NHase by NO produced in situ by rat brain NO-synthase. A: difference UV-vis spectra of 14 μM NI1 NHase in 50 mM Hepes buffer pH 7.4, in the presence of 44 nM rbNOS, and 100 μM L-arginine at 28°C recorded every 2 min after addition of 300 μM NADPH to start the reaction. B: absolute UV-vis spectra of 14 μM NI1 NHase before (——) and after (— — —) 11 min incubation in the presence of rbNOS under physiological conditions as in A; then after 10 min photoactivation(• • •). Curves in agreement with two independent experiments.

3.4. Coupling of NHase and NO-synthase

It has been proposed that N771 NHase could be nitrosylated in vivo by a bacterial NO-synthase [9]. The following experiments have been done to test this hypothesis. NI1 NHase (28 μM iron) was incubated in aerobic conditions with purified rat brain NO-synthase (rbNOS) (44 nM) in the presence of its substrates, L-arginine and NADPH, and cofactors (see Section 2), that are required for NO formation. Under these conditions, the charge transfer band of NHase completely disappeared after 11 min (Fig. 5A). Identical results were obtained with BR312 NHase. The rate of NO production by rbNOS was determined by the HbFe(II)O_2 assay [19] and found to be 3 μM NO per min; thus, after 10 min, the amount of NO (30 μM) produced by rbNOS and required for complete inactivation of NHase well corresponded to about one equivalent of NO per NHase iron. Moreover, a 10 min photoactivation of NI1 or BR312 NHase inactivated by NO-synthase was sufficient to completely restore their charge transfer band (Fig. 5B).

4. Discussion

The aforementioned results show that purified, active BR312 NHase is completely inactivated by 1–2 equivalents (per iron) of NO, and fully reactivated after photoirradiation. They also show that there is a clear relationship between NHase activity and the presence of a charge transfer band around 700 nm (Fig. 2B). NHase inactivation by NO always occurred with the disappearance of this band and its replacement by a 370 nm band; its photoactivation is associated with the reappearance of the ≈700 nm band. In fact, the UV-vis spectrum of NO-inactivated BR312 NHase is identical to that previously reported for the in vivo inactive state of N771 NHase which seems to contain NO as an iron ligand [9]. This result is easily understandable as BR312 and N771

NHases have an identical amino-acid sequence [15,16]. Moreover, during the preparation of this manuscript, a paper appeared about the resonance Raman spectra of N771 NHase both in its in vivo inactive, NO-containing state and in its photoactivated state [25]. It showed that addition of NO to the photoactivated NHase restored the state characterized by an UV-vis band at 370 nm and the 592 and 570 cm^{-1} Raman bands that are assigned to Fe-NO stretching and bending vibrations.

Our results provide new data on the interaction of NO with NHases as they establish for the first time that (i) another iron-containing NHase, NI1 NHase, which only exhibits a 50% sequence homology with BR312 NHase [17], is also inactivated by NO and photoactivated in a manner almost identical to BR312 NHase, and (ii) NO has a high affinity for the NHase Fe^{III} centre, similar to the one for $HbFe^{III}$ (Fig. 3), and inactivates NHase in a very efficient manner. In fact, addition of 1–2 equivalents of NO relative to NHase iron is sufficient to completely inactivate the enzyme (Fig. 1). Moreover, complete inactivation of NHase was obtained by coupling of NHase with rbNOS, and in situ formation of only one equivalent of NO (per NHase iron). This result demonstrates that NHase is very efficiently inactivated by NO, even produced under physiological aerobic conditions in the presence of species able to trap NO, such as O_2. This efficient coupling between NHase and rbNOS strongly supports the proposition that N771 NHase would be inactivated in vivo in the dark by NO produced by a NO-synthase that could exist in *Rhodococcus* sp. N771.

BR312 and NI1 NHases undergo 2–3 cycles of inactivation by NO and photoreactivation without significant loss of enzymatic activity (Fig. 4). Actually, the progressive loss of activity observed after more than three cycles was obtained by using four equivalents of NO per NHase iron in the inactivation step. As only one equivalent seems sufficient for this inactivation (in experiments using NO-synthase), it is likely that the irreversible denaturation observed after more than three cycles is due to NO in excess. More cycles could probably be obtained after careful removal of NO.

Finally, the efficient coupling between NHase and NO-synthase and the ability of different NHases to be inactivated by NO and then photoactivated for several consecutive cycles without loss of enzymatic activity suggest that NO and light could play an important role in the regulation of iron-containing NHases in microorganisms.

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