Kinetics of CO binding and CO photodissociation in *Pseudomonas stutzeri* *cd*₁ nitrite reductase: probing the role of extended N-termini in fast structural relaxation upon CO photodissociation

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*cd*₁ nitrite reductase from *Pseudomonas stutzeri* is a di-haem-containing enzyme, comprising a *c*-type haem and a *d*-type haem. Studies with the highly related *cd*₁ nitrite reductase of *Pseudomonas aeruginosa* have established that this enzyme undergoes fast (microsecond) and global structural relaxation upon CO photodissociation from the reduced enzyme. A key difference between the *Ps. aeruginosa* and *Ps. stutzeri* enzyme is the absence of a flexible N-terminal extension in the *Ps. stutzeri* enzyme. In *Ps. aeruginosa* *cd*₁ nitrite reductase the N-terminal extension wraps around the second subunit of the homodimer and with Tyr¹⁰ stabilizing a water molecule co-ordinated to the *d*₁-haem. Given the intimate association of the N-terminal extension with the *d*₁-haem, we hypothesized that the presence of the N-terminal extension likely contributes to the fast structural reorganization seen during photodissociation of CO from the reduced enzyme. In the present study we have investigated the kinetics of CO association and CO photodissociation of *Ps. stutzeri* *cd*₁ nitrite reductase (which lacks the N-terminal arm seen in the *Ps.*

aeruginosa enzyme) to probe the role and influence of the N-terminal arm in the fast global structural reorganization seen with *Ps. aeruginosa*. Surprisingly, we find that *Ps. stutzeri* *cd*₁ nitrite reductase also undergoes fast structural reorganization during CO photodissociation. We also show, in stopped-flow experiments, that the kinetics of CO binding and dissociation with reduced *Ps. stutzeri* *cd*₁ nitrite reductase are similar to those observed with *Ps. aeruginosa* enzyme, thus ruling out a major role for the N-terminal flexible arm found in *Ps. aeruginosa* in the kinetics of these processes. Our data indicate that global structural reorganization following CO photodissociation is an intrinsic property of the haem domains in *cd*₁ nitrite reductases. The absence of an N-terminal extension, as in the *Ps. stutzeri* *cd*₁ nitrite reductase, does not lead to loss of global structural reorganization following CO photodissociation.

Key words: CO recombination, flash photolysis, haem protein, structural dynamics.

INTRODUCTION

Denitrification is the enzymic pathway that allows facultative anaerobic bacteria to use nitrate instead of oxygen as an electron acceptor for energy production (for a review, see [1]). Nitrite reductase (NiR) plays an important role in this process as it catalyses the one-electron reduction of nitrite to NO:

These nitrite reductase proteins fall into two major classes depending on the redox centres they employ: one class contains copper and the other contains two haem cofactors, a *c*-type haem and a unique *d*₁-haem (*cd*₁ NiR) (for reviews, see [2–4]). The latter class of enzymes are soluble homodimers (subunit molecular mass 60 kDa); following *c*-haem reduction from external donors, such as cytochrome *c*₅₅₁ and azurin [5–7], electrons are passed sequentially to the *d*₁-haem, which is the site of substrate binding and catalysis [6–8].

*cd*₁ NiRs have attracted a considerable amount of attention over the years. Recent interest has focused on the high-resolution crystal structures of *cd*₁ NiR from *Paracoccus pantotrophus* (*Pp*-NiR) [9,10] and *Ps. aeruginosa* (*Pa*-NiR) [11,12]. Extensive

spectroscopic and kinetic studies [6–8], and more recently site-directed-mutagenesis experiments [13], have complemented the structural work. These studies have highlighted a number of intriguing features of *cd*₁ NiRs. First, they are bi-functional enzymes and can perform the four-electron reduction of oxygen to water (albeit with low efficiency), a chemical reaction far removed from their physiological role. Secondly, they are also prone, under certain conditions, to severe product inhibition, as NO binds to the ferrous *d*₁-haem with high affinity. Finally, although these enzymes share a common function and overall structure, they show a number of structural variations and employ different haem ligands in the oxidized state.

The structures of *Pp*-NiR [9,10] and *Pa*-NiR [11,12] show that *cd*₁ nitrite reductase comprises three domains: a flexible N-terminal extension or 'arm', a *c*-domain (which contains the *c*-haem) and a *d*-domain (which houses the *d*₁-haem). However, the position of the N-terminal arm in the two proteins is not the same. In *Pp*-NiR the arm interacts with the *d*-domain of the same subunit, and one of the residues in the N-terminal arm (Tyr²⁵) is the sixth ligand of the ferric *d*₁-haem. In contrast, the N-terminal arm of *Pa*-NiRs wraps itself around the *d*-domain of the second subunit (rather than the subunit to which it is linked covalently).

Abbreviations used: NiR, nitrite reductase; *Pa*-NiR, *Pseudomonas aeruginosa* NiR; *Ps*-NiR, *Pseudomonas stutzeri* NiR; *Pp*-NiR, *Paracoccus pantotrophus* NiR.

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Although Tyr¹⁰ (equivalent to Tyr²⁵ in *Pp*-NiR) is in close proximity to the *d*₁-haem, in this case it does not form the sixth ligand to the ferric *d*₁-haem; the sixth ligand is provided by a hydroxide ion. On the basis of sequence-comparison studies, *Ps*-NiR lacks the N-terminal segment altogether [14]. There are thus clear structural differences between the *cd*₁ nitrite reductases from different species, which might have functional consequences. These differences are found (i) in the presence/absence of an N-terminal extension, (ii) the nature of subunit-subunit interaction involving the N-terminal arm and (iii) the identity of ligands at the haem centres. It is noteworthy that in both redox states, *Pa*-NiR and *Ps*-NiR have His and Met as their *c*-haem axial ligands, whereas the ferric *c*-haem in *Pp*-NiR has two His ligands, but upon reduction one of the His residues is exchanged for a Met residue. These differences are additional to those mentioned above concerning the nature of the ligands to the ferric *d*₁-haem: in *Pp*-NiR there are two protein ligands (Tyr²⁵ and His), whereas *Pa*-NiR has a hydroxide ion and His¹⁸² is the distal ligand.

Recently it was demonstrated that, when CO is photodissociated from the ferrous *d*₁-haem of *Pa*-NiR, fast (micro-second) structural relaxations are observed [15]. These relaxations were interpreted as arising from global structural changes rather than specific movements of amino acids within the distal pocket of the *d*₁-haem. As this class of protein is known to exhibit a number of structural variations, it was of interest to probe to see whether the fast structural relaxations are a common property of *cd*₁ NiRs. An important question is whether the presence of the N-terminal arms, and the mode by which they interact with the *d*₁-haem, account for the global structural changes observed with *Pa*-NiR. With this in mind, we have investigated the reaction of *Ps*-NiR (which lacks the N-terminal arm) with CO and the behaviour of this enzyme upon CO photodissociation. We show here that *Ps*-NiR exhibits structural rearrangement following CO photodissociation, and that the relaxation kinetics of the transiently populated species are only modestly different from those observed with *Pa*-NiR. Our data indicate, therefore, that the fast structural rearrangement observed with *Pa*-NiR is not directly attributable to the presence of the N-terminal arm and its mode of interaction with the *d*₁-haem. The fast structural relaxation following CO photodissociation is therefore an intrinsic property of the *d*₁-haem and/or *c*-haem domain, although the presence of the N-terminal arm may modulate the kinetics of this structural change.

MATERIALS AND METHODS

Preparation of protein samples

Ps-NiR was purified as described in [16]. The preparation was judged pure when the oxidized enzyme had an absorbance ratio (A_{411}/A_{280}) of ≥ 1.1 . The protein concentration was determined using $\epsilon_{411} = 141 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for the oxidized protein (which refers to the concentration of active sites).

Stopped-flow and equilibrium experiments

Both the stopped-flow and equilibrium CO-binding experiments were carried out under anaerobic conditions in 100 mM potassium phosphate, pH 7.0, containing 1 mM EDTA at 20 °C. For stopped-flow experiments, protein samples were prepared by reducing the enzyme (5 μM) with sodium ascorbate (1 mM final concentration) in a tonometer. Following complete reduction the protein sample was rapidly mixed with buffer containing various concentrations of CO (400–50 μM), ensuring that all reactions were carried out under pseudo-first-order conditions. The re-

action was monitored at 460 nm using an Applied Photophysics (Leatherhead, Surrey) stopped-flow instrument. Time courses were analysed using the computer program MATLAB (The Math Works Inc., South Natick, MA, U.S.A.). For equilibrium CO-binding experiments, *Ps*-NiR (8.3 μM) was placed in a capped cuvette and the gas phase was excluded. Buffer containing 1 mM CO was introduced with a Hamilton syringe through the septum of the cuvette and the absorbance spectrum of the sample after each addition was measured using a JASCO Corporation (Hachioji City, Tokyo, Japan) V-550 spectrophotometer.

Laser-photodissociation experiments

Laser-CO-photodissociation experiments were carried out using an Applied Photophysics LKS.60 flash-photolysis instrument. The Applied Photophysics LKS.60 offers tunable-wavelength excitation pulses, by using the third harmonic (355 nm) of a Continuum Surelite 1-10 Nd YAG laser and a manually adjusted Surelite Optical Parametric Oscillator (OPO) system. The apparatus was configured to deliver 440 nm pulses. Kinetic transients were recorded at 420, 450, 460, 480, 500 and 620 nm. The maximum photolysis achieved (15%) was estimated by comparing the static absorbance difference between reduced *Ps*-NiR and the *Ps*-NiR-CO complex with the absorbance changes monitored in the laser experiments. All reactions were performed at ambient temperature and under anaerobic conditions. Protein samples (12 μM), contained in 50 mM potassium phosphate buffer, pH 7.0, were placed in anaerobic cuvettes (pathlength 1 cm). *Ps*-NiR was reduced with 300 μM ascorbate and 1 mM CO was subsequently introduced into the cuvette to form the *Ps*-NiR-CO complex.

RESULTS AND DISCUSSION

Binding of CO

The binding of CO to reduced *Ps*-NiR was monitored in the stopped-flow apparatus at various concentrations of CO. At all

Figure 1 Kinetic transients recorded at 460 nm after rapidly mixing reduced *Ps*-NiR (5 μM) with buffer containing various concentrations of CO

The observed rate constants were determined by fitting the data to a single exponential. Trace A, $[\text{CO}] = 200 \mu\text{M}$; trace B, $[\text{CO}] = 100 \mu\text{M}$; the reaction was carried out under anaerobic conditions in 100 mM potassium phosphate buffer, pH 7, containing 1 mM ascorbate and 1 mM EDTA, at 20 °C. The inset graph shows a plot of the observed rate constant against CO concentration, giving a second-order association rate constant of $5.3 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ and a dissociation rate constant of 0.27 s^{-1} .

Figure 2 Equilibrium titration of *Ps*-NiR (8.3 μ M) with CO

The reaction was carried out under anaerobic conditions in 100 mM potassium phosphate buffer, pH 7, containing 1 mM ascorbate and 1 mM EDTA at 20 °C. CO-saturated buffer (1 mM CO) was injected with a gas-tight syringe and the spectrum recorded after every addition. Spectrum A is the fully reduced protein, while spectrum B is the 100%-reduced CO-bound protein. The inset graph is a plot of the absorbance change at 460 nm as a function of CO concentration. The continuous line is a fit of the data to a hyperbola giving a K_d of 22 μ M.

CO concentrations the reaction was monophasic and the time courses were fitted to a single exponential rate equation (Figure 1). A plot of the observed rate constant against the CO concentration (Figure 1, inset) yields a second-order association rate constant (k_{on}) of $5.3 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ and a dissociation rate constant (k_{off}) of 0.27 s^{-1} . Both of these kinetic parameters are similar to those reported for *Pa*-NiR (association rate constant of $2 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ and a dissociation constant of 0.12 s^{-1}). One study on the reaction of reduced *Pa*-NiR with CO suggested that binding of CO was co-operative [17]. To investigate whether *Ps*-NiR bound CO co-operatively, equilibrium-titration experiments were performed (Figure 2). A plot of the absorbance change at 460 nm as a function of CO added (Figure 2, inset) and a double-logarithmic plot (not shown) of the data provided no evidence for cooperative binding (Hill coefficient, h , = 0.91). A dissociation constant, K_d , of 22 μ M was calculated for the CO complex with the reduced enzyme. There is a small discrepancy between the kinetic and equilibrium data; from the kinetic measurements a K_d of 50 μ M was calculated, which is approximately twice as large as the corresponding value calculated from the equilibrium-binding experiments. Of interest is the finding that the affinity for CO in *Ps*-NiR is less than that observed in *Pa*-NiR, which most likely reports on local structural differences in the d_1 -haem domain.

Photodissociation of CO

Upon photodissociation of CO from fully reduced *Ps*-NiR, fast (microsecond) kinetic transients are observed (Figure 3A). At all wavelengths studied (420, 450, 460, 480, 500 and 620 nm) the absorbance changes were best described as monophasic and were fitted to a single exponential process with a rate constants of 5700 s^{-1} . The sign of the amplitude was consistent with the kinetic difference spectrum reported by Wilson et al. [15]. Following the fast kinetic processes a monophasic slower transient was observed which could be fitted to a single exponential yielding a rate of 8 s^{-1} at 1 mM CO. This phase has been assigned to relaxation of the transient back to the dark state. This

assignment is consistent with the sign of the amplitude change reporting on the re-binding of CO. The observed rate is also very similar to that expected for the re-binding of CO to *Pa*-NiR [15]. At the end of this rebinding phase the absorbance of the protein returned to the pre-trigger level, corresponding to full recovery of the CO-bound protein.

Following photodissociation of CO, the fast kinetic process cannot be due to geminate rebinding of CO to the d_1 -haem, as the absorbance changes observed are not consistent with this process (for example compare Figures 3A and 3B; the two kinetic processes have amplitudes of opposite sign). As with previous studies on *Pa*-NiR [15], we assign these fast kinetic transients to global structural rearrangements of the protein that are triggered by CO photodissociation. Inspection of Figure 3(A) indicates that immediately after the laser pulse the protein populates an unstable structural intermediate (denoted by the 'X' in Figure 3A), which then relaxes to the conformation of the reduced unliganded protein. At all the wavelengths studied with *Ps*-NiR, the kinetic intermediate has a spectrum similar to that observed with *Pa*-NiR. Clearly *Ps*-NiR undergoes structural perturbations similar to those described previously with *Pa*-NiR. However, there are a number of differences between the two proteins. For *Ps*-NiR at all wavelengths studied, the structural relaxations occur monotonically with a rate of 5700 s^{-1} , whereas in *Pa*-NiR the kinetic transients were biphasic ($k_{fast} 30000 \text{ s}^{-1}$ and $k_{slow} 2500 \text{ s}^{-1}$ [15]). It is important to draw the distinction at this point between a *single* monophasic processes and *multiple* processes that occur with similar time constant. We clearly cannot distinguish between the two possible scenarios, and one must acknowledge, therefore, the possible population of multiple structural states during the relaxation process. A complex global relaxation process involving multiple species (each with individual time constants) might thus account for the observed differences in kinetic behaviour (i.e. monophasic versus biphasic) between the two enzymes. Additionally, we cannot exclude unequivocally a minor and very rapid ($> 100000 \text{ s}^{-1}$) absorption increase (Figure 3A) following photolysis, owing to limitations caused by recovery of the detection system following the laser flash. Clearly if such a phase does exist, it would be substantially

Figure 3 Transient observed at 460 nm after CO photodissociation from reduced *Ps*-NiR (12 μ M)

Experimental conditions were 50 mM potassium phosphate, pH 7, containing 300 μ M ascorbate. **(A)** Time course observed over a millisecond time scale. The absorbance (Abs) of the CO-bound protein is set at zero, and the laser pulse is indicated by the vertical line. Immediately after the pulse the protein populates an unrelaxed intermediate denoted by the 'X'; this species then relaxes in a monophasic process with a rate constant of 5700 s^{-1} . **(B)** Transient observed on a second time scale after CO photodissociation; the continuous line represents a fit of the data to a single exponential rate equation yielding a rate constant of 8 s^{-1} at 1 mM CO.

faster than the fast phase (k_{fast} 30000 s^{-1}) observed in the *Pa*-NiR cd_1 nitrite reductase. In the case of *Pa*-NiR, the complexity of the structural relaxations were taken to indicate that the structural relaxation might not be due to the movement of specific amino acid residues within the d_1 -haem distal pocket, but rather more global structural alterations. It is important to bear in mind that there is no counterpart in *Ps*-NiR of Tyr¹⁰ present in *Pa*-NiR (which is located close to the reduced d_1 -haem), owing to the lack of an N-terminal arm in *Ps*-NiR. This important structural variation might contribute to the observed kinetic differences between the two enzymes. The probable lack of the fast phase in *Ps*-NiR, which is observed in *Pa*-NiR, suggests that this phase may be directly related to the presence of the N-terminal extension in *Pa*-NiR. A possible explanation is that the fast phase observed in *Pa*-NiR is due to structural perturbations of the arm, perhaps involving Tyr¹⁰, which is located in the d_1 -haem distal pocket. The data indicate that the spectrum of the kinetic intermediate populated in both proteins is similar, but it appears that the kinetic pathway the protein takes on moving from the kinetic (metastable structural) intermediate to the fully reduced equilibrium structure likely differs between *Pa*-NiR and *Ps*-NiR.

Our work with the *Ps. stutzeri* enzyme indicates that the fast global structural changes observed in the cd_1 nitrite reductases

following CO photodissociation are not dependent on the presence of an extended N-terminal arm. The origin of these structural changes is clearly of interest in the understanding the mechanism of CO release and recombination in cd_1 nitrite reductase. Recent flash-photolytic studies of the *Pp*-NiR also reveal that fast structural changes occur following photodissociation of the CO complex and that the kinetic transients describing these changes are biphasic [18]. Moreover, the slower of the two phases is accompanied by proton uptake from solution. Further insights into possible changes occurring during photodissociation have come from crystallographic studies of the unligated and the NiR-CO complex [18]. These studies reveal changes in the puckering of the d_1 -haem in addition to rearranged hydrogen-bonding networks and solvent organization in the haem pocket. The use of Tyr²⁵ of the N-terminal arm as the sixth ligand to the d_1 -haem in oxidized enzyme in *Pp*-NiR might be expected to contribute to the structural relaxations following CO photodissociation. It is important to note that in *Pa*-NiR the corresponding residue (Tyr¹⁰) does not co-ordinate directly to the d_1 -haem, but instead stabilizes a water molecule that is co-ordinated to the haem. Nevertheless, the N-terminal extension is clearly intimately involved in d_1 -haem interaction in *Pa*-NiR and, as with *Pp*-NiR, is expected to contribute to the fast structural changes following CO photodissociation. Our work with the *Ps. stutzeri* enzyme suggests that this is indeed the case, owing to loss of the faster of the two phases seen in the *Pa*-NiR and *Pp*-NiR enzymes. The challenge now is to identify the nature of these global changes and to relate them to catalytic events in the family of cd_1 nitrite reductases.

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