Spectrophotometric activity micro-assay for pure and recombinant cytochrome P450-type nitric oxide reductase[1]

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Short title

Nitric oxide reductase activity assay

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Subject category

Enzymatic assays and analyses

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Abstract

Nitric oxide reductase (NOR) of the P450 oxidoreductase family accepts electrons directly from its cofactor, NADH, to reduce two nitric oxide (NO) molecules to one nitrous oxide molecule and water. The enzyme plays a key role in removal of radical NO produced during respiratory metabolism while applications in bioremediation and biocatalysis have been identified. However, a rapid, accurate and sensitive enzyme assay has not yet been developed for this enzyme family. In this study, we optimised reaction conditions for the development of a spectrophotometric NOR activity micro-assay using NOC-5 for the provision of NO in solution. We also demonstrate that the assay was suitable for the quantification and characterization of P450-type NOR. The K_m and k_{cat} kinetic constants obtained by this assay were comparable to the values determined by gas chromatography, however with improved convenience and cost efficiency, effectively by miniaturisation. To our knowledge, this is the first study to present the quantification of NOR activity in a kinetic micro-assay format.

Keywords: nitric oxide reductase, enzyme assay, NADH

Introduction

Nitric oxide reductases (NOR) have been isolated from prokaryotes [1, 2, 3] and eukaryotes [4]. Despite the differences in enzyme structure and molecular mechanism of NO reduction, these enzymes catalyse the same reaction [3]. In general, NOR are involved in denitrification pathways and nitric oxide (NO) detoxification [4, 5, 6]. In fungi, NO is an intermediate

product of nitrate reduction during respiration [7] and, since it is a reactive nitrogen species, it has the capacity to damage cellular components at high concentrations [8].

NOR activity is currently determined by gas chromatography [5], amperometric assays [9], stopped-flow rapid scan spectroscopy [10] and conventional UV spectrophotometry [11, 12, 13, 14]. The development of a micro-titre spectrophotometric assay is limited by the requirement for bubbling of NO gas through the reaction medium as source of substrate. Thus, to date, there is no report of a simple miniaturised micro-spectroscopic assay for characterizing this class of enzymes.

NO is a small diatomic gaseous molecule with a very short half-life and fast diffusion rate [15]. Previously, in assays developed for monitoring of NOR activity, NO was provided in the assay by saturating the reaction mixture with NO gas [10, 13, 16, 17]. The development of NO releasing reagents or zwitterionic polyamine such as NOC-5 [18], has allowed for the quantification of NO from NOC-5 in solution with an assay that followed the reduction of NO through spectrophotometric quantification of NADH [19].

Kaya *et al.* (2004) [19] demonstrated the linearly proportional relationship between the oxidation of NADH to NAD^+, with the release of NO from NOC-5, but did not develop a kinetic assay for NOR activity using this principle, the primary aim of this study. Nakahara *et al.* (1993) [10] determined the stoichiometry of NO reduction by NOR as 2:1:1 for NO:NADH:N_2O. Therefore, for each NADH oxidized to NAD^+, two molecules of NO are converted to N_2O (refer Equation 1).

$$2\ NO + NADH + H^+ \rightarrow\ N_2O + H_2O + NAD^+ \quad \text{(Equation 1)}$$

There is an increasing interest in NOR as a class of enzymes, due to its potential for industrial applications such as the removal of NO from the environment [20, 21]. Due to the current lack of an appropriate kinetic assay for monitoring NOR activity, we have developed a fast and reliable method for the quantification and kinetic characterization of NOR. This assay utilizes the monitoring of NADH as a suitable mechanism for measuring the reduction of NO by NOR in real-time.

Materials and methods

Materials

A NOR solution from *Aspergillus oryzae* was purchased from Wako Chemical GmbH (Germany). For the expression of *A. oryzae* derived NOR, Anor, from the pET-28a vector Novagen (USA), *E. coli* BL21(DE3) (F^-,*dcm, ompT*$^-$*, hsd*S(r_B^-,m_B^-), *gal*, λ (DE3) was obtained from Stratagene (USA). *E. coli* cell lysis was carried out using detergent based YPER solution (Thermo Scientific) and DNase I (Bio-Rad, USA). The protease inhibitor cocktail was purchased from Sigma-Aldrich (Germany). The nickel affinity resin, NTA MagReSyn™, was a gift from ReSyn Biosciences (South Africa). The magnetic separator from Invitrogen (Life Technologies, USA) was used.

Reactions were performed in flat bottom 96 well micro-titre plates from Greiner Bio One (Germany) and 384 well micro-titre plates from Genetix (now Molecular Devices, England). Assay reagents including NADH and buffer components were purchased from Sigma-Aldrich (Germany) and NOC-5 ([3-(2-hydroxy-1-(methylethyl)-2-nitrosohydrazino)-1-propanamine]) was purchased from Merck-Millipore (South Africa). Greiner Bio One UV plates were used for the spectrophotometric quantification of NOC-5 at 250 nm. The optical density was

measured in a BioTek Powerwave HT (United States of America) UV VIS micro-titre plate reader.

Methods

Assay development

With the intention of creating a reliable kinetic method for the quantification of NOR, we set out to establish the assay parameters starting with the NADH concentration and the determination of the light path in the micro-titre plates (96 and 384 wells). Optimum pH, temperature and buffer conditions were determined and then the subtrate concentration range that could be obtained from the NO-donor reagent, NOC-5. Finally, the dynamic enzyme concentration range was determined under these optimal conditions for enzyme activity determination. For the validation, and as an example of an application of this assay, the kinetic parameters of the commerial and a recombinant preparation of NOR were determined.

NADH concentration

NADH has an absorption maximum of 340 nm with a molar extinction coefficient, ϵ, of 6.22 $mM^{-1}.cm^{-1}$ [22, 23]. The detection limit of the BioTek Powerwave HT micro-titre plate reader at 340 nm was determined. The length of the light path in a 96 well and in a 386 well micro-titre plate, with 150 µl and 70 µl total reaction volume respectively, was calculated using the Beer-Lambert relationship between the extinction coefficient and path length. The light path for the 150 µl volume in a 96 well micro-titre plate was calculated as 0.35 cm and for the 70 µl volume in the 384 well micro-titre plate was calculated to be 0.47 cm.

Reaction temperature and pH

It is well known that enzyme activity is affected by temperature and pH as well as other factors. Thus, it was important to characterise NOR activity at various pH values and temperature values. The enzyme activity of the commercial NOR was determined at room temperature and 37˚C.

The substrate for NOR, NO, was obtained from the decomposition of NOC-5, which is pH dependent [24]. Therefore, the NOC-5 decomposition rate at various pH values was determined first. NOC-5 was observed to be stable at high pH and thus a 200 mM NOC-5 stock solution was prepared in 50 mM NaOH. To obtain the maximum rate of NO release, NOC-5 was allowed to decompose in Britton-Robinson buffer (50 mM sodium phosphate dibasic, 50 mM boric acid, 33 mM citric acid and 50 mM TRIS; with pH adjustment using potassium hydroxide) within a pH range of 4.2 to 8.6. The decomposition of 1 mM NOC-5 was monitored at 37˚C for 30 minutes in a UV transparent micro-titre plate at 250 nm.

Thereafter, an activity profile of the commercial NOR over the pH range 4.2 to 8.6 in Britton-Robinson buffer was established. To eliminate the NO release rate from NOC-5 decomposition as a determinant of NOR activity at different pH values, a 20 mM NOC-5 solution was pre-incubated in Britton-Robinson buffer pH 5 for 1 minute at 37˚C, before it was added to the buffered enzyme solution for the activity determination at a particular pH. This was done to ensure that each sample contained the same initial amount of NO.

Assay Buffer

Although sodium phosphate buffer at pH 7.2 is frequently used for NO reduction assays [9, 10, 13, 19], there is no report of the evaluation of various buffering agents that could be found for NOR activity assays. Due to this lack of comparison we evaluated a variety of

potentially suitable buffers for assay at pH 6, the apparent optimum pH for NOR activity. In consideration of the addition of base stabilised NOC-5, a relatively high buffer concentration of 200 mM was chosen for the assay to prevent an alkaline pH shift of the assay reagents. The following buffering agents were chosen for evaluation: sodium phosphate; MES (2-(*N*-morpholino)ethanesulfonic acid); HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); TRIS (tris(hydroxymethyl)aminomethane); and TEA (triethanolamine).

Estimation of substrate in solution

The introduction of NOR's substrate has previously been conducted by bubbling of NO gas into the enzyme reaction mixture, but this approach is cumbersome for high throughput assays. The release of this volatile substrate from a NO donor into solution was therefore preferred for the development of a kinetic assay. For the estimation of the effective substrate concentration in solution, the release rate of NO from NOC-5, as well as the extent of other contributing factors that could reduce NO concentration, had to be elucidated. Therefore, the NO release rate of NOC-5 was determined by two methods: calculated from the six order polynomial described below *(A*) and the enzymatic reduction of NO to N_2O by NOR *(B*).

NO concentration in the assay may be affected by several factors; including the pH dependent release of NO from NOC-5, oxidation of NO by oxygen, as well as the rate of diffusion of volatile NO [18, 25, 26]. Due to these variables, the determination of NO concentration from NOC-5 decomposition required more sophisticated analysis. The relationship of NO release and its subsequent fate was previously described as a six order polynomial equation [25, 26]:

NO in solution = NO release rate – NO/O_2 oxidation – NO diffusion

$$d[NO]/dt = e_{NO}k_1[NOC\text{-}5]_0^{(-kt)} - 4k^*[NO]^2[O_2] - (k_La/V)[NO] \qquad \text{(Equation 2)}$$

The first term of equation 2 describes the rate of release of NO from the NO donor, which was characterized as first order release kinetics in deoxygenated solution. The NO release rate = $e_{NO}\ k_1\ [NOC\text{-}5]_0^{(-kt)}$, where k_1 (min^{-1}) is the first-order decomposition constant (obtained by decrease in absorbance of the NOC-5 at 250 nm as it spontaneously releases NO [24, 25] and $[NOC\text{-}5]_0$ the initial NOC-5 concentration. The stoichiometric ratio of NO release, e_{NO}, is theoretically equal to two [18]. However, this ratio is dependent on the reaction conditions and may vary [26, 27]. The time taken for the NOC-5 decomposition is assigned as t (min).

The oxidation kinetics of NO in aqueous solutions are represented by the second term in equation 2, which further contributes to the uncertainty of the exact concentration of NO in solution. This factor accounts for the reaction of NO with oxygen in solution to form nitrite ions. The stoichiometry of this reaction is generally expressed as: $4NO + O_2 + 2H_2O \rightarrow 4H^+ + 4NO_2^-$ [28, 29]. The rate of nitrite formation is described by the rate constant, k*, of 2.4 x $10^6\ M^{-2}.s^{-1}$ between 35 to 37˚C [30].

The third component in equation (2) is the volumetric mass transfer coefficient that describes the diffusion of NO from the liquid phase into the gas phase. Lewis & Deen (1994)[30] proposed that the NO diffusion from liquid into gas was the main process in which NO was removed from the reaction mixture [30].

Method *(A)* involved the calculation of NO in solution by solving only the first term of equation 2, i.e. the rate of release of NO (mM NO/min): $d[NO]/dt = e_{NO}k_1[NOC\text{-}5]_0^{(-kt)}$, where e_{NO} was assumed to be 2 or less, where k_1 was the average of the rate constants determined and NOC-5 concentration at time t=0. This equation does not include the potential loss of NO by oxidation or diffusion, which was assumed to be negligible due to NO consumption by NOR as soon as the NO is produced. For the determination of the rate constant of NOC-5 decomposition, k_1, the rate of decomposition for NOC-5 (concentrations

between 0.016 and 2 mM) in 200 mM TEA buffer (pH 6) was recorded at 250 nm for 30 minutes at 37˚C in a 96 well UV micro-titre plate as described before [24, 25]. The first-order decomposition rate constant, k_1, was calculated from the gradient (-m) generated by a plot of ln (A_0-A∞) with time, where A_0 is absorbance at t=0 and A∞ is the absorbance at the time when no further decrease in absorbance is observed [24, 25]. The average of all the rate constants was applied to calculate the rate of NO release.

For method *(B)*, the enzymatic determination of NO (as demonstrated by Kaya *et al.* (2004); [19]), a constant concentration of NOR (0.3 µM) was allowed to reduce NO generated from varying concentrations of NOC-5 (0 to 8.6 mM) in 200 mM TEA buffer pH 6 at 37˚C. The rate of reduction was estimated by the following the rate of NADH consumption monitored by the decrease in absorbance at 340 nm for 5 minutes in a 384 well plate.

Lower limit of detection and dynamic range

On completion of the optimisation of the various assay parameters, an evaluation of assay linearity, lower limit of detection (sensitivity) and dynamic range was undertaken. NOR activity was measured by a decrease in absorbance at 340 nm (NADH) over 5 minutes in a 384 well micro-titre plate with increasing concentrations of a commercial NOR preparation (0.02 to 0.9 µM) to establish the dynamic range of the NOR activity assay. NOC-5 concentration was added in excess to the reaction mixture to ensure that NOC-5 did not limit the NADH consumption. Thus, the enzyme reaction contained 2 mM NOC-5 and 1 mM NADH in 200 mM TEA pH 6 buffer at 37˚C.

For validation as a micro-assay, the assay was repeated in 96 well (150 µl) and 384 well micro-titre plate (70 µl), containing increasing concentrations of a recombinant NOR (expressed in *E. coli* BL21 (DE3)).

Assay Reproducibility

The reproducibility of the NOR activity assay was established by repeating the assay on three consecutive days with reagents prepared on each day with the optimal reaction condition (2 mM NOC-5 and 1 mM NADH in 200 mM TEA pH 6). A 20 mM NOC-5 stock solution (in 50 mM NaOH) was allowed to release NO for one minute at 37˚C in Britton-Robinson buffer pH 5 before it was added to the reaction mixture. The intention of this pre-incubation of NOC-5, was to pre-empt the oxidation of NADH by NOC-5 and to ensure excess NO in solution. A NOR dilution series was prepared from the commercial NOR solution and the NO reduction was initiated by the addition of the assay reagents. The NADH consumption was monitored for 5 minutes at 37˚C.

Kinetic characterisation of NOR in micro-assay

In the past numerous enzymes with NOR activity have been isolated from various sources [12, 31, 32]. This indicates the general interest in the NO reducing enzymatic reaction. For the characterization of new isoforms of P450-type NO reducing enzymes, a fast and reliable assay is required to determine the kinetic constants. A non-linear regression analysis for single ligand binding (SigmaPlot 10.0, Systat Software Inc. Germany) and Lineweaver-Burk analysis (reciprocal of initial velocity (v_0) and substrate concentration [S]), were used to calculate the enzyme's kinetic parameters using a 0.14 µM concentration of the commercial NOR solution. This methodology was also applied to determine the kinetic constants of two recombinant NOR preparations. The kinetic constants were compared to the values reported by Kaya *et al.* (2004) [19].

Expression and purification of recombinant Anor in E. coli

The amplification of the coding sequence of Anor, termed *nicA*, was ligated into *Sal*I and *Noc*I restriction sites on pET-28a with a C-terminal His-tag sequence configuration. The recombinant plasmid, pET-*nicA* was electroporated into chemically competent *E. coli* BL21 (DE3). Transformed *E. coli* BL21 (DE3) containing pET-*nicA* were termed *E. coli* BL21[*nicA*]. To purify and characterise recombinant Anor, an overnight starter culture of *E. coli* BL21[*nicA*] was used to inoculate 500 ml Luria broth containing 50 $\mu g.ml^{-1}$ kanamycin and the culture was grown at 37˚C and 190 rpm orbital shaking to a final cellular abundance measuring with an optical density of 0.4 at 600 nm. At this point, IPTG was added to a final concentration of 0.5 mM for induction of gene expression. The temperature was then reduced to 30˚C. The cells were harvested after 18 hours of growth, lysed and the released histidine tagged protein was recovered by magnetophoretic MagReSyn™ NTA affinity purification medium. The concentration of the recombinant Anor preparation was determined according to the method described by Omura & Sato, (1964) [33]. The NOR activity was determined by the NOR activity assay with the optimised reaction conditions. The assay was repeated without NOC-5 for all the samples. This value was subtracted from the NADH oxidation rate obtained in the presence of NOC-5. This was done to normalise for NADH consuming enzymes in the cell lysate.

Results

Optimum conditions for NOR activity assay

The optimum conditions for the quantification of NOR were established as 2 mM NOC-5 and 1 mM NADH in 200 mM TEA buffer (pH 6) at 37˚C. The NOR activity at 37˚C was 24.4 µM

NO/min which was approximately double that at 28˚C (12.3 µM NO/min), thus all subsequent assays were performed at 37˚C.

The pH dependent NOC-5 decomposition is shown in Figure 1. The most suitable NOC-5 decomposition rate was observed at pH 5. Neither low (pH 4) or high pH (8 to 9) was suitable for NOC-5 decomposition. At low pH (pH 4), NO was released at an extremely fast rate and NO gas formation (in the form of bubble formation) interfered in the spectrophotometric assay, whereas at pH 5, the NOC-5 decomposition rate was completed after 90 seconds without interference. The NOC-5 decomposition was more gradual in Britton-Robinson buffer pH 6 with a maximum rate after 5 minutes. No apparent NOC-5 decomposition took place at pH 8 or 9.

The pH profile of NOR demonstrated maximum enzyme activity at pH 6.2, with high activity also detected at pH 6.6 (Figure 2). A variety of buffers at pH 6 and 200 mM concentration were subsequently evaluated for their effect on the enzyme assay. Although the choice of buffer had little effect on the activity, increased activity was observed in TEA and HEPES buffer (refer Table 1).

Substrate concentration and availability

The accurate quantification of NO concentration in solution is essential to determine the kinetic parameters of nitric oxide reducing enzymes. Due to several variables influencing the effective concentration of NO in solution after its release from NOC-5 decomposition, accurate estimation of NO concentration presented a challenge. This study attempted to determine the NO concentration by (*A)* a mathematical calculation of NO release from NOC-5 which was compared to (*B)* a biochemical determination from enzymatic NO reduction by NOR.

A) Mathematical calculation of substrate concentration

The decomposition of NOC-5 was determined by spectrophotometric measurement at 250 nm from which the decomposition rate constant, k_1, was calculated as described by Maragos *et al.* (1991) [24]. The calculated NO release did not include the NO oxidation reaction, or the volumetric mass-transfer coefficient. This method for NO concentration only applies for a closed system, and was chosen since it best simulated the enzymatic consumption of NO after its formation. For this mathematical computational method, the equation was solved by applying the variables as indicated in Table 2. The initial NOC-5 concentration, $[NOC\text{-}5]_0$, and the calculated NO release rate (μM NO/min) generated a direct linear correlation with an equation where $y= 0.147x$ ($R^2= 1$). The average of the decomposition rate constant, k_1, was used in the subsequent determinations for rate of release of NO. The knowledge of the NOC-5 decomposition rates was essential for the substrate optimization studies as it allowed for the estimation of NO concentrations.

B) Enzymatic quantification of substrate

Kaya *et al.* (2004) [19] indicated that NOR isolated from *Aspergillus oryzae* was suitable for the quantification of NO in solution. The rate of enzymatic reduction of NO is dependent on the available NO in solution. In this study, we set out to investigate whether the decrease in absorbance of NADH presented a direct correlation to the rate of release of NO from NOC-5. A linear increase in the NO reduction rate was observed between 0 and 1.5 mM NOC-5 (excess enzyme). These results support the findings of Kaya *et al.* (2004) [19] that NOR could potentially be adapted to quantify NO in solution.

A very close correlation between the two methods of NO estimation indicated that the release rate of NO from NOC-5 can be determined by either the enzymatic quantification ($y= 87.5x$; $R^2= 1$; refer Table 3) or by applying the decomposition rate constant ($e_{NO}\ k_1\ [NOC\text{-}5]_0$

e^{-kt} (y= 87.3x; R^2= 1; refer Table 3). The close correlation indicates that the non-enzymatic reduction of NO by oxidation and diffusion is likely to be negligible. The decomposition stoichiometry of NOC-5 is theoretically 2 or less [18] and it has been noted that this may be less in different reaction conditions [24]. In this study, this stoichiometrical ratio, e_{NO}, was ≈ 1.19.

Lower limit of detection and dynamic range for NOR activity assay

Initially the NOR activity was determined with decreasing concentrations of a commercial NOR solution to estimate the range of enzyme concentrations that could be detected. The results indicated that a linear range from 0.04 to 0.9 µM could be quantified, correlating to between 1.5 and 160 µM NO/min (refer Figure 3 A). The assay was verified for quantification with recombinant NOR in two plate formats, 96 wells or 384 wells with reaction volumes of 150 µl and 70 µl respectively. For the 384 well plate, a linear increase in NOR activity from 14 to 308 µM NO/min (y= 2.269x; R^2= 0.991) was observed, whereas the 96 well plate presented a linear dynamic range from 10 to 282 µM NO/min (y= 3.378x; R^2= 0.981) as shown in Figure 3 B. Overall, a linear dynamic range in excess of one order of magnitude was noted for the enzymatic assay.

Assay Reproducibility

In order to determine assay reproducibility, the assay was reproduced over multiple days. Inter-well variability and day to day variability were less than 10% within the dynamic range of the assay (refer Figure 4). Higher variability was observed at the higher NOR concentrations. The experimental set confirms the assay is reliable (R^2 equal to 0.964).

Quantification of Recombinant Anor purification yield

The quantification NOR yield from *E coli* BL21[*nicA*] presents an example for an application of the NOR activity assay. The optimal assay conditions were used to determine the purification yield which is presented in Table 4. In consideration of NADH oxidation in the cell lysate (L), the NADH consumption rated determined in the absence of NOC-5 was subtracted from the NOR activity. Furthermore, the lysate of *E. coli* BL21 (DE3) without a pET-28a vector was included to quantify other nitric oxide related redox activity naturally present in *E. coli*, such as nitric oxide reductases [1] and nitric oxide dioxygenases [8]. This background NO reducing activity accounted for 24% when compared to total NOR activity in a cell lysate (L), indicating that an estimated 19% of Anor was found in the unbound fraction (UB). NOR activity from real biological samples were quantified with the NOR activity assay. However, NOR activity from cell lysates and unbound fraction required corrections for NADH-NO catalysing enzymes.

Kinetic characterisation of NOR

The enzyme kinetics was derived from the initial reaction rates with increasing substrate concentrations to determine the single-substrate reaction with the Michealis-Menten kinetic model [34]. The focus of kinetic characterisation was the determination of substrate affinity (K_m), maximal velocity (V_{max}), specificity constant (V_{max}/K_m) and enzyme efficiency (K_{cat}/K_m).

The kinetic characterization of commercial NOR solution (Anor from *A. oryzae*) and recombinant NOR preparation (Anor from *E. coli* BL21[*nicA*]) aimed to establish the affinity of NOR for NO under the described assay condtions, to confirm the potential application in enzyme characterisation and to demonstrate the versatility of the assay. Kinetic constants

for NOR from *A. oryzae* have previously been reported [19], providing a viable comparison for the kinetic constants obtained with the NOR activity assay.

At first, however, NOR activity assay and the NO estimation in solution was validated by the determination of kinetic constants of the commercially prepared NOR by measuring the initial velocities at increasing substrate concentrations. Lineweaver-Burk double reciprocal plot (LB) and non-linear regresssion analyis (N-L) were employed to calculate the kinetic constants (Table 5). The Michealis-Menten constant (K_m) and the maximal reaction velocity (V_{max}) were comparable for both NO estimation methods (enzymatic vs. mathematical) as well as for the two different calculations methodologies (LB vs. L-N) which presented only a 0.5% difference in K_m values. The NO estimation method did not present any differences in the maximal velocity values (632 µM NO/min) and presented only 0.04% difference the two calculation methods (632 vs. 658 µM NO/min). This indicates that the NO estimation for the NOR activity assay was suitable for the determination of kinetic characterisation.

The kinetic constants (K_m) and maximal velocities (V_{max}) reported by Kaya and his colleagues ($Anor^A$) [19], the wild-type Anor (commercial Anor preparation, $Anor^B$) as well as two different recombinant enzyme preparation ($Anor^C$ and $Anor^D$) are shown in Table 6. The kinetic constant, K_m, from the wild-type Anor determined by the NOR activity assay ($Anor^B$) deviated only by a marginal 11% from the K_m determined by gas chromatography ($Anor^A$). $Anor^B$ presented a slower catalytic rate (4700 NO/min) which might be due to compromised enzyme integrity caused by extend storage duration. These results indicate that the NOR activity assay is suitable for the determination of substrate affinity as well as measuring the enzyme integrity.

The kinetic characterisation described for the commercial preparation was repeated for the recombinant Anor. A higher substrate affinity (lower K_m) was measured for $Anor^C$ and $Anor^D$

which were expressed in and purified from *E. coli* BL21[*nicA*]. The maximum velocity (V_{max}) for Anor^{D} deviate only by a marginal 3% from the wild-type Anor^{A}. Similarly for the catalytic turnover (k_{cat}) of the recombinant enzymes was within the same order of magnitude when compared to the wild-type Anor^{A}. The determination enzyme efficiency [35, 36] and specificity constant (V_{max}/K_m) [35, 37, 38] was included for a more comprehensive characterisation of the recombinant Anor. A higher specificity constant (V_{max}/K_m) as well as improved enzyme efficiency (K_{cat}/K_m) highlights that Anor expression in *E. coli* might have altered NOR's interaction with its substrate. The NOR activity presents sufficient sensitivity for the detection of changes in enzyme kinetics presenting a second application for this assay.

Discussion

This study set out to develop a simple kinetic assay to quantify NOR activity from a P450-type NOR. To achieve this, various assay variables were investigated. The oxidation of NADH was chosen as a colorimetric "readout" for the quantification of NO reduction. NADH has a strong extinction coefficient and a wide dynamic range with suitable sensitivity. The optimal reaction mixture and conditions were 1 mM NADH, 2 mM NOC-5 in 200 mM TEA buffer pH 6 at 37˚C. NOR activity was evaluated in several types of buffers with little variation on enzyme activity, indicating the potential versatility of this enzyme assay.

The optimization of substrate concentration and estimation of solution phase NO required a more in depth analysis. The NO concentration in solution derived from the decomposition of NOC-5 has previously been described as a six order polynomial (Equation 2). However, we demonstrate that in the presence of enzyme, the rate of NO release is the major variable determining NO concentration, and that alternate factors influencing NO concentration are negligible. To our knowledge this is the first study that presents the relationship between the

quantification of NO with NOR and its correlation to a mathematical estimation of NO. We propose that this approach may be suitable for the development of other assays utilising rate dependant substrate release agents.

We demonstrate a linear dynamic range in excess of one order of magnitude. This assay is currently suitable for quantification of NOR activity, however, the lack of sensitivity indicates that the assay is unlikely to be suitable for potential application in the quantification of NO in biological solutions. Furthermore, the use of NADH based assays is complicated by the presence of alternate NADH consuming enzymes as well as NO reduction in crude samples such as cell lysates as observed in the purification of recombinant Anor. The assay demonstrated high inter-well reproducibility and low day to day variability. This assay does not require laborious techniques or expensive equipment, and is therefore proposed as a reliable method for the quantification of NOR activity and suitable for kinetic characterisation of a commercial Anor preparation as well as of recombinant Anor. We further demonstrated that the NOR activity assay presented sufficient sensitivity for the detection of altered enzyme affinity in the recombinant Anor preparations.

The miniaturisation of this kinetic assay allows for higher throughput application such as the screening of Anor secreting *A. niger* D15 transformants and the quantification of NOR activity after Anor immobilisation.

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Tables

Table 1.

NO reduction rate by 0.07 µM NOR at 37˚C in various buffers (200 mM).

Buffer	µM NO/min	SD[a]
$NaPO_4$	182.9	11.1
MES	191.4	11.3
TRIS-HCl	210.4	14.7
TEA	250.9	4.3
HEPES	289.8	9.5

[a] standard deviation of triplicates

Table 2.

Kinetic parameters for mathematical NO estimation in TEA buffer, pH 6.0.

$[NOC\text{-}5]_0$	k_1 (per min)[a]	µM NO/min[b]
2	0.202	294.3
1	0.174	145.7
0.5	0.235	72.6
0.25	0.242	36.1
0.125	0.250	17.9
0.064	0.254	9.1
0.031	0.272	4.3
0.016	0.102	2.0
average	0.216	

$[NOC\text{-}5]_0$ in mM

[a] k_1= gradient (-m) which was determined from ln (A_0-A_{30}) [25]

[b] NO release rate=e_{NO} k_1 $[NOC\text{-}5]_0$ $^{e\text{-}kt}$, where e_{NO}= 2, t= 5 min [26]

Table 3.

The calculated NO release rate from enzymatic NO quantification and mathematical estimation.

	μM NO/min	
	y=87.5x	y= 87.3x
mM NOC-5	Enzymatic NO reduction rate	NO release rate*
8.58	750.8	748.9
4.29	375.4	374.5
2.5	218.8	218.2
1.25	109.4	109.1
0.67	58.6	58.5
0.4	35.0	34.9
0.2	17.5	17.5
0	0.0	0.0

*e_{NO}=1.19

Table 4.

NOR activity purified from *E. coli* BL21[*nicA*].

		Total protein	Total activity	Specific activity	Yield
Source	Sample	mg	Units	Units.mg^{-1}	%
E. coli BL21[*nicA*]	L	4.2	38.7	9.3	100
	UB	3.0	16.5	5.6	43
	E	1.0	36.1	35.2	93
Negative control	L	3.5	9.4	2.7	

L-lysate, UB- unbound, E-eluate
Unit= 1 μmol NO.min^{-1} [19]

Table 5.

Kinetic constants of 0.14 μM NOR from *A. oryzae*.

NO quantification method	LB[a] plot		N-L[b]	
	K_m	V_{max}	K_m	V_{max}
enzymatic	209	632	222	658
equation	208	632	221	658

[a] Lineweaver-Burk plot
[b] Non-linear regression analysis
K_m and V_{max} in μM NO and μM NO.min^{-1}, respectively

Table 6.

Kinetic characterization of Anor purified from *E. coli* BL21[*nicA*].

NOR	μM NOR	K_m μM NO	V_{max} μM NO.min^{-1}	k_{cat} NO.min^{-1}	k_{cat}/K_m min^{-1}.μM^{-1}	V_{max}/K_m
Anor^A	0.026	97	269	10567	109	2.8
Anor^B	0.037	117	342	9364	80	2.9
Anor^C	0.140	222	658	4700	21	3.0
Anor^D	0.028	197	352	12571	64	1.8

Anor^A and Anor^B: recombinant Anor
Anor^C: commercial wild-type Anor solution
Anor^D: reported values of wild-type Anor [19]

Figure legends

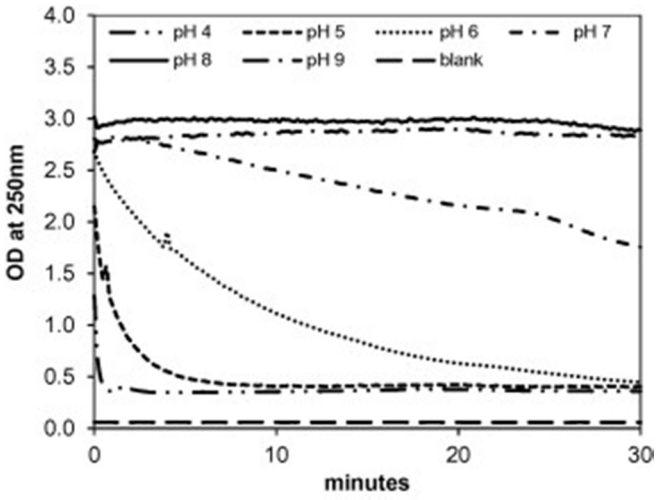

Figure 1. The pH profile of NOC-5 decomposition. A stock solution of 5 mM NOC-5 was prepared in 50 mM NaOH and added to Britton-Robinson buffer to a final concentration of 1 mM. NOC-5 decomposition was monitored at 250 nm in a UV micro-titre plate at 37˚C for 30 minutes. The blank sample contained only Britton-Robinson buffer.

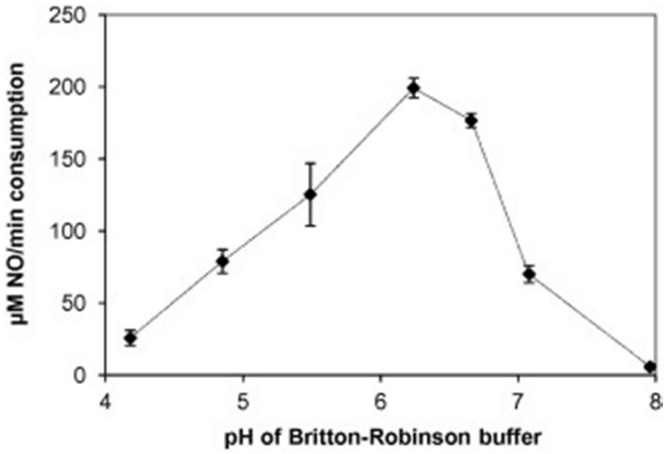

Figure 2. pH profile of NOR activity. The enzyme reaction contained 0.07 μM NOR solution, 1.7 mM NOC-5 and 1 mM NADH in Britton-Robinson buffer with various pH's. The reaction was monitored for 5 minuates at 37˚C. The decrease in absorbance at 340 nm was correlated to NADH consumption which was converted to μM NO/min consumption.

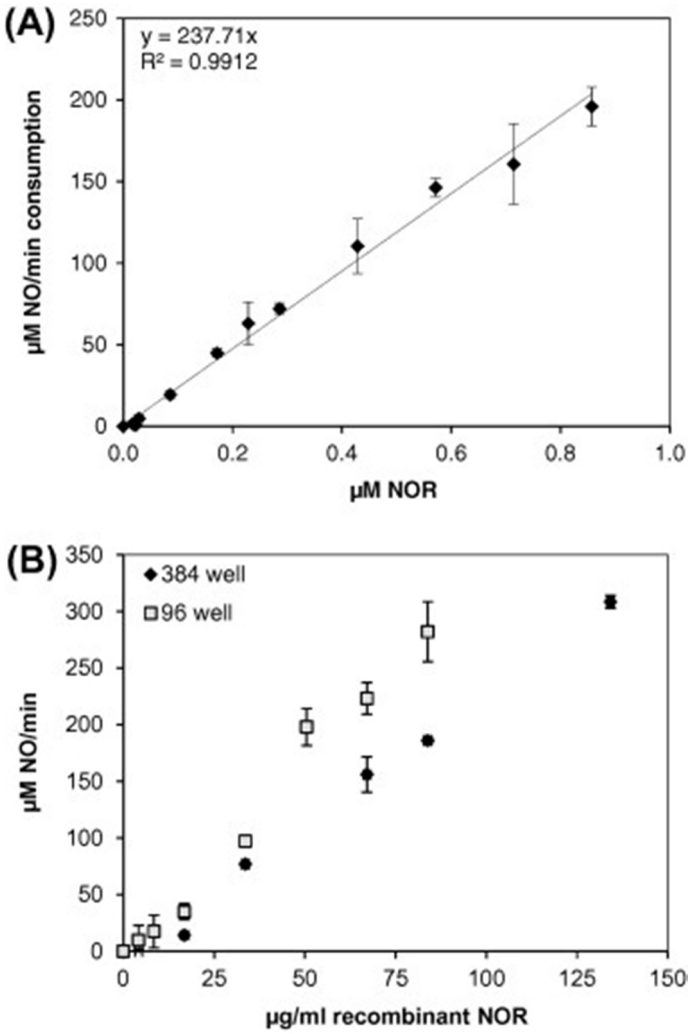

Figure 3. Linear dynamic range of NOR activity assay. A) The NOR activity was determined with increasing concentrations of the commercial NOR in a 384 well plate. In B) The NOR activity was determined with increasing enzyme concentrations of recombinant NOR protein content in 384 well plate and in 96 well plate. The reaction mixture contained 2 mM NOC-5, 1 mM NADH and 200 mM TEA buffer (pH 6) and was performed at 37˚C for 5 minutes. The enzyme activity (µM NO/min consumption) was calculated from the NADH consumption rate.

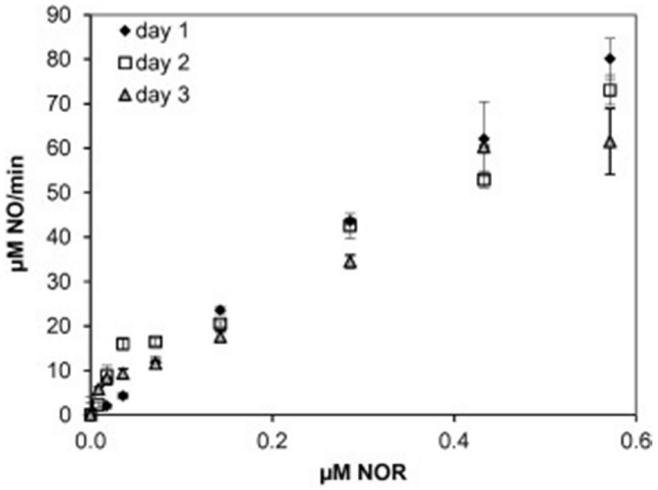

Figure 4. Reproducibility of NOR activity assay. The linear relationship of NOR concentration and NO reduction was measured on three consecutive days. The reaction mixture contained 5 µl commercial NOR at various concentrations, 2 mM NOC-5 and 1 mM NADH in 200 mM TEA pH 6. The NADH consumption rate was monitored for 5 minutes at 37˚C from which the enzyme activity (µM NO/min consumption) was calculated.