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Spectrophotometric activity microassay for pure and recombinant cytochrome P450-type nitric oxide reductase

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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 the removal of radical NO produced during respiratory metabolism, and 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 optimized reaction conditions for the development of a spectrophotometric NOR activity microassay using NOC-5 for the provision of NO in solution. We also demonstrate that the assay is 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, but with improved convenience and cost efficiency, effectively by miniaturization. To our knowledge, this is the first study to present the quantification of NOR activity in a kinetic microassay format.

Nitric oxide reductases (NORs) have been isolated from prokaryotes [1–3] and eukaryotes [4]. Despite the differences in enzyme structure and molecular mechanism of nitric oxide (NO) reduction, these enzymes catalyze the same reaction [3]. In general, NORs are involved in denitrification pathways and NO detoxification [4–6]. 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 [11–14]. The development of a microtiter 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 miniaturized microspectroscopic 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 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 polyamines 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. [19] demonstrated the linearly proportional relationship between the oxidation of NADH to NAD^+ and 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. [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 to Eq. (1)):

$$2NO + NADH + H^+ \rightarrow N_2O + H_2O + NAD^+. \quad (1)$$

There is an increasing interest in NORs as a class of enzymes, because of their potential for industrial applications such as the removal of NO from the environment [20,21]. Because of 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 NORs. This assay utilizes the monitoring of NADH as a suitable mechanism for measuring the reduction of NO by NORs 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),

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Escherichia coli BL21(DE3) (F^-, *dcm*, *ompT*$^-$, *hsdS*(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, MagReSyn™ NTA, 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 microtiter plates from Greiner Bio One (Germany) and 384-well microtiter plates from Genetix (now Molecular Devices, England). Assay reagents including NADH and buffer components were purchased from Sigma–Aldrich 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 (USA) UV–Vis microtiter plate reader.

Assay development

With the intention of creating a reliable kinetic method for the quantification of NORs, we set out to establish the assay parameters starting with the NADH concentration and the determination of the light path in the microtiter plates (96 and 384 wells). Optimum pH, temperature, and buffer conditions were determined and then the substrate 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 commercial 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 microtiter plate reader at 340 nm was determined. The length of the light path in a 96-well and in a 386-well microtiter plate, with 150 and 70 µl total reaction volume, respectively, was calculated using the Beer-Lambert relationship between the extinction coefficient and the path length. The light path for the 150-µl volume in a 96-well microtiter plate was calculated as 0.35 cm and for the 70-µl volume in the 384-well microtiter 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 characterize 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 (tris(hydroxymethyl)aminomethane), 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 min in a UV-transparent microtiter 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 various pH values, a 20 mM NOC-5 solution was preincubated in Britton-Robinson buffer, pH 5, for 1 min 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. Because of 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-stabilized 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, and TEA (triethanolamine).

Estimation of substrate in solution

The introduction of NOR's substrate has previously been conducted by bubbling 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]. Owing 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]:

$$\text{NO in solution} = \text{NO release rate} - \text{NO/O}_2 \text{ oxidation} - \text{NO diffusion},$$
$$d[\text{NO}]/dt = e_{NO}k1[\text{NOC} - 5]_0^{(-kt)} - 4k^*[\text{NO}]^2[\text{O}_2] - (k_\text{L}a/V)[\text{NO}]. \quad (2)$$

The first term of Eq. (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 = $e_{NO}k_1[\text{NOC-5}]_0^{(-kt)}$, where k_1 (min^{-1}) is the first-order decomposition constant (obtained by decrease in absorbance of NOC-5 at 250 nm as it spontaneously releases NO [24,25]) and $[\text{NOC-5}]_0$ the initial NOC-5 concentration. The stoichiometric ratio of NO release, e_{NO}, is theoretically equal to 2 [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 is represented by the second term in Eq. (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