Talanta 78 (2009) 910–915

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Reductive decomposition of a diazonium intermediate by dithiothreitol affects the determination of NOS turnover rates

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ARTICLE INFO

Article history:
Received 24 June 2008
Received in revised form 24 December 2008
Accepted 30 December 2008
Available online 23 January 2009

Keywords:
Griess assay
Diazonium
Dithiothreitol
Nitric oxide synthase
Turnover
External calibration
Standard addition

ABSTRACT

Accurate determination of nitrite either as such or as the breakdown product of nitric oxide (NO) is critical in a host of enzymatic reactions in various settings addressing structure–function relationships, as well as mechanisms and kinetics of molecular operation of enzymes. The most common way to quantify nitrite, for instance in nitric oxide synthase (NOS) mechanistic investigations, is the spectrophotometric assay based on the Griess reaction through external standard calibration. This assay is based on a two-step diazotization reaction, in which a cationic diazonium derivative of sulfanilamide is formed as intermediate before the final absorbing azo-product. We show that this intermediate is very sensitive to reducing agents that may be transferred from the reaction media under investigation. The interaction of this vital intermediate with the reducing agent, dithiothreitol (DTT), which is widely used in NOS reactions, is characterized by both electrochemical and spectroscopic means. The effect of DTT on the performance of external calibration, both in sample recovery studies and in actual NOS reactions, is presented. Finally an alternative method of standard additions, which partially compensates for the accuracy and sensitivity problems of external calibration, is proposed and discussed.

1. Introduction

Determination of trace amounts of nitrite is crucial in a number of mechanistic and kinetic investigations addressing structure–function of enzymes. Examples include investigations addressing mechanisms and kinetics of the molecular function of enzymes such as Nitrite Reductases (NiR) [1] and nitric oxide synthases (NOS) [2]. In the latter case, accurate determination of nitrite (NO_2^-) as a breakdown product of nitric oxide (NO) is of particular importance in current investigations, which aim to understand the molecular function of NOS enzymes. Nitric oxide synthases are heme enzymes that catalyze the in vivo synthesis of nitric oxide, a diatomic molecule that was found to mediate numerous physiological processes and is involved in the development of a host of pathological states [3]. NO is involved in vasodilation, neurotransmission, cytotoxicity, and cytoprotective processes [4,5]. It is biosynthesized by enzymatic oxidation of one terminal guanidine-nitrogen of the amino acid L-arginine through the *N*-hydroxy-L-arginine intermediate, yielding L-citrulline as a co-product (Scheme 1).

Although several methods have been developed to determine the NOS turnovers [6–8], NO is often quantified in the form of nitrite (NO_2^-), a stable breakdown product of NO in aerobic reaction media. Various methods are employed to quantify nitrite accurately especially in biological samples [9–13]. The most commonly used technique to quantify nitrite in NOS mechanistic investigations is using a spectrophotometric assay based on the Griess reaction through a standard calibration curve [14,15]. This assay is based on a two-step diazotization reaction in which acidified nitrite produces a nitrosating agent which reacts with sulfanilamide to produce the diazonium derivative. The diazonium ion is then coupled to *N*-1-napthylethylenediamine dihydrochloride (NED) to stoichiometrically form the final azo-product which absorbs at 540 nm (Supporting information, Scheme S1). The Griess assay is widely used to determine nitrite in a variety of biological and experimental matrices such as plasma, serum, urine, and tissue culture media [16–18]. However, serious accuracy problems may arise in the presence of interfering agents, especially if these are not taken into account during the external calibration. It has been already reported that the NOS enzyme itself spectrally interferes with the final product of Griess assay [19]. The NADPH, an essential cofactor in NOS reaction, is also a known interferent in the Griess assay [20]. John Moody and Shaw recently published a reevaluation study of the Griess assay in terms of the extent of interference brought by nicotinamide nucleotides in the assay medium [21]. A prior review by Fox and Suhre documented interferences by a number of agents including thiols [22].

Accurate quantification of nitrite is critical for reliable determination of NOS turnover and other kinetic aspects; it thus can affect

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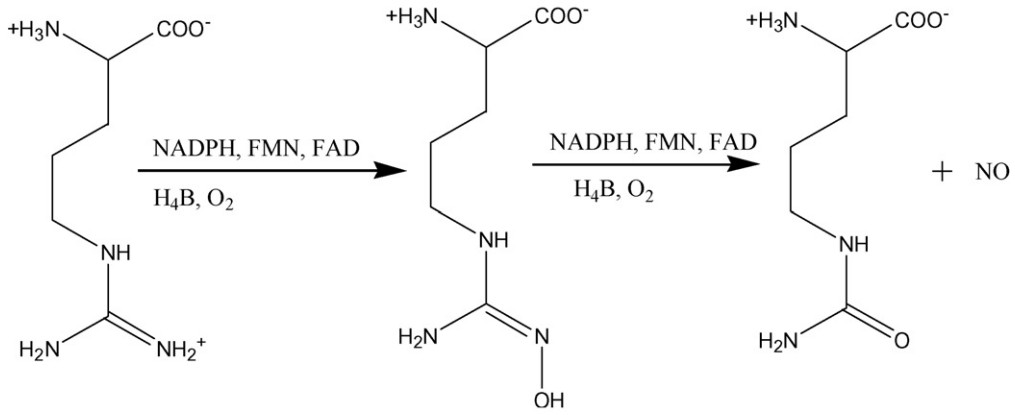
doi:10.1016/j.talanta.2008.12.074



Scheme 1. Enzymatic synthesis of nitric oxide by NOS enzymes; the reaction uses L-arginine as a substrate and oxygen as co-substrate. NADPH, FAD, FMN, and tetrahydropterin are also necessary for the NOS function.

all derived mechanistic/kinetic interpretations. The cationic diazonium intermediate that forms during the first step of the Griess assay is vulnerable towards most reducing agents in the assay medium, especially dithiothreitol (DTT), which is widely used in NOS reactions. The interaction between diazonium intermediate and DTT, especially when present in high concentrations (0.5 mM or higher), results in a critically low yield for final azo-compound, which seriously affects the sensitivity of the method. This, in turn, leads to serious limitations for nitrite determination especially in the low concentration range (i.e. 2.5–10 μM), where important mechanistic information is usually derived through measurement of initial rates.

In this work, we probe the nature of critical interaction of the diazonium intermediate with DTT as a reducing agent using electrochemical and spectrophotometric techniques. The study also explores how DTT concentration affects the sensitivity of the Griess assay for nitrite determination, even when matrix correction (i.e. using DTT in standards) is used in the external calibration method. The study further investigates possibilities to minimize the matrix difference to achieve high accuracy and sensitivity using standard addition method.

2. Experimental

2.1. Reagents

All the chemicals used were of analytical grade. Nanopure deionized water (specific resistance > 18.2 Ω cm) used in all experiments was supplied by a Barnstead water purification system. All working solutions of nitrite were prepared using 0.1 M standard nitrite solution. Dithiothreitol and NADPH were purchased from Sigma. The Griess reagent kit was purchased from Promega (Madison, WI) and was used following the technical instructions provided. Briefly, the analyte solution is incubated with sulfanilamide first for 10–15 min, followed by the addition of NED (10–15 min), and the absorbance of the final azo dye (λ_{max} at 540 nm) is recorded. It is worth mentioning the concentration of the Griess ingredients in the stock solutions of the kit. Based on the chemical specifications on the Promega kit, the concentration of sulfanilamide solution is 58 mM while the NED solution is at 3.9 mM. These concentrations ensure that the Griess assay ingredients are always in excess compared to the nitrite analyte.

2.2. Apparatus

UV–vis absorbance spectra were recorded on Agilent 8453 spectrophotometer. Spectra were collected between 400 and 650 nm. Cyclic voltammetry was performed in a standard three-electrode cell using a BAS100W electrochemical workstation (Bioanalytical Systems Inc.). A gold working electrode (CHI, Area = 0.0314 cm^2) was polished with alumina slurry (successively with 0.3 and 0.05 μm), and cleaned in ultrasound bath in deionized water.

2.3. Electrochemical measurements

All electrochemical measurements were carried out in nitrogen purged 0.05 M NaCl solution. The solutions were stirred between the cyclic voltammetric experiments; all potential are reported versus the Ag/AgCl reference electrode. After each scan in solutions of diazonium ions, the working electrode was cleaned with piranha solution (70% H_2SO_4 and 30% H_2O_2) and polished as described above.

2.4. Procedure for calibration curves

The Griess assay used widely is a two-step process. First the analyte aliquot is incubated for 15 min with an excess sulfanilamide to generate the diazonium cation. NED (in excess of the diazonium cation formed) is then added to the assay medium and the color is allowed to develop. Absorbance is recorded after exactly 15 min of reaction time. Calibration curves were constructed for the range of 2.5–15 μM nitrite; standards were prepared using 0.1 M nitrite stock solution. 500 μL-aliquots of standards are incubated for 15 min each with 100 μL sulfanilamide and 100 μL NED. Deionized water is added up to 1.00 mL and absorbance measurement taken at 540 nm.

To evaluate the matrix effect, samples for recovery studies are prepared by mixing various amounts of nitrite and DTT (from 1 mM working solutions) and diluting with deionized water to 1.00 mL. 500 μL aliquots of each unknown were incubated for 15 min with 100 μL sulfanilamide followed by 100 μL NED and diluted with deionized water to 1.00 mL. The absorbance is then measured and the nitrite amount is quantified using a suitable calibration curve.

2.5. Procedure for standard addition method

Samples containing two different concentrations of nitrite (3 and 6 μM, prepared from a 0.1 M stock solution just as we did for the regular calibration method) in the presence of three representative concentrations of DTT (0.0, 0.4, and 1.0 mM) were prepared, giving a total of six test solutions. The total volume of each sample analyzed was 3.00 mL. The range of nitrite concentration in these working samples was selected based on actual ranges used in NOS reaction investigations. Each test solution was divided into