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Nitroxidative chemistry interferes with fluorescent probe chemistry: Implications for nitric oxide detection using 2,3-diaminonaphthalene

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ABSTRACT

Simultaneous production of nitric oxide (NO) and superoxide generates peroxynitrite and causes nitroxidative stress. The fluorometric method for NO detection is based on the formation of a fluorescent product from the reaction of a nonfluorescent probe molecule with NO-derived nitrosating species. Here, we present an example of how nitroxidative chemistry could interact with fluorescent probe chemistry. 2,3-Naphthotriazole (NAT) is the NO-derived fluorescent product of 2,3-diaminonaphthalene (DAN), a commonly used NO-detecting molecule. We show that NO/superoxide cogeneration, and particularly peroxynitrite, mediates the chemical decomposition of NAT. Moreover, the extent of NAT decomposition depends on the relative fluxes of NO and superoxide; the maximum effect being reached at almost equivalent generation rates for both radicals. The rate constant for the reaction of NAT with peroxynitrite was determined to be $2.2 \times 10^3\ M^{-1}\ s^{-1}$. Further, various peroxynitrite scavengers were shown to effectively inhibit NO/superoxide- and peroxynitrite-mediated decomposition of NAT. Taken together, the present study suggests that the interference of a fluorometric NO assay can be originated from the interaction between the final fluorescent product and the formed reactive nitrogen and oxygen species.

1. Introduction

Nitric oxide (NO) is an important, ubiquitous free radical in biological systems. This simple diatomic gas molecule is in no way simple in its chemistry and biological actions. NO mediates various physiological functions via delicately regulated and controlled biosynthetic pathways [1]. Once formed intracellularly, the biotransformation of NO is complicated because of its radical nature [2,3]. On the one hand, NO can immediately react with several metalloproteins and exert its direct biological actions [1]; on the other, NO could react with oxygen and oxidative-stress-derived reactive species, which leads to complex nitroxidative chemistry presumably responsible for the indirect action of NO [1,4,5]. In particular, when NO and superoxide are spatially and temporally coexisting, a potent oxidant, peroxynitrite, is formed instantaneously [5,6]. Under this circumstance, the biological effect of NO is less intuitively predictable because of complex interactions between multiple reactive species and biomolecules [7,8].

A quantitative determination of NO is essential for better understanding the chemistry and biology of NO, but this is not an easy undertaking – given that NO is highly reactive and short-lived. Several methods including electrochemical, chemiluminescent, colorimetric and fluorometric detections have been routinely used for determining the amount of NO generated in both chemical and biological systems [9,10]. Besides its simplicity, sensitivity, and convenience of use, one of the advantages of using a fluorescent compound (probe) to detect NO levels is its potential for allowing continuous real-time measurements [11], although a truly quantitative one remains a challenge. In the case of measuring cellular NO production, a nonfluorescent probe upon cellular uptake would "report" the presence of NO by reacting with NO-derived nitrosating species to form a fluorescent end product [12]. The formation of fluorescent products is a kinetic process involving complex chemistries associated with both nitroxidative species and the probe molecule, which would render it difficult, if not impossible, to conduct a true quantitative determination. Indeed, the fluorescent probes generally do not react directly with NO; they either react with the nitrosating species (e.g., N_2O_3) derived from the autoxidation of NO, or react indirectly with NO via a reactive-nitrogen-species-mediated oxidative process, especially when superoxide or other radicals are also present, that generates free-radical intermediates of the probe molecule [13,14]. The nonspecific nature of the radical intermediates would further react with oxygen or various biomolecules (e.g., antioxidants) to

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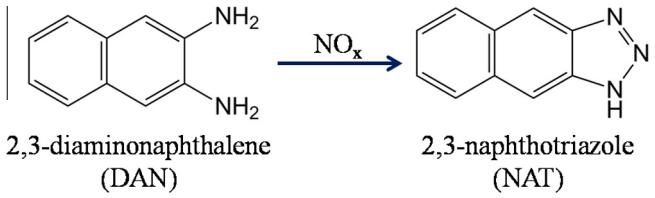

Scheme 1. The rationale of NO detection using 2,3-diaminonaphthalene (DAN). Nonfluorescent DAN reacts with NO-derived nitrosating species (NO_x), forming a fluorescent product (NAT).

produce multiple reactive species that would in turn affect NO levels. While this is a well-recognized, potential problem associated with the use of fluorescent NO probes [12], an issue yet to be addressed is the one linked with the stability of the fluorescent product formed during continuous measurements. This is nontrivial because NO-derived nitroxidative species can be very reactive, possibly making the fluorescent products one of their targets. For example, peroxynitrite, a very reactive molecule produced from NO/superoxide co-generation, is known to react with numerous molecular targets [5,6]. Recently, there have been vigorous pursuits of specific probes for peroxynitrite [15–21]; however, to the best of our knowledge, the reaction of peroxynitrite with the fluorescent product of an NO probe has not been reported.

2,3-Diaminonaphthalene (DAN) has been frequently used in detecting NO levels and nitrosation status in cells and in chemical systems [13,22–26]. The detection is based on the formation of a triazole product (NAT) upon reaction of DAN with reactive nitrogen species (NO_x, Scheme 1, [27,28]). Previously, we unexpectedly observed that SIN-1, a donor of both NO and superoxide, facilitates the fluorescent decay of NAT [29], which prompts us to investigate in detail the putative interaction between the fluorescent probe and the NO/superoxide-derived nitroxidative chemistry. In the present study, we demonstrate that NO/superoxide co-generation mediates the chemical decomposition of NAT. In particular, we show that the extent of NAT decomposition depends on the relative fluxes of NO and superoxide, with a maximum effect occurring at a flux ratio near 1. The result is consistent with the premise that peroxynitrite and its derived reactive species react with NAT, thereby causing its decomposition. Notably, the role of peroxynitrite is confirmed by a kinetic analysis and by experiments in which peroxynitrite scavengers were used.

2. Materials and methods

2.1. Materials

N-[4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl]-1,3-propanediamine (Spermine NONOate; SPENO), 3-morpholinosydnonimine (SIN-1), cytochrome c (horse heart), catalase (bovine liver), glutathione, L-ascorbic acid, uric acid, diethylenetriaminepentaacetic acid (DTPA), and 2,3-diaminonaphthalene (DAN) were obtained from Sigma (St. Louis, MO). 4,4′-[Azobis(oxymethylene]bis-benzoic acid (SOTS-1) and peroxynitrite were purchased from Cayman (Ann Arbor, MI). Potassium phosphate (monobasic) and dimethyl sulfoxide (DMSO) were obtained from J.T. Baker (Phillipsburg, NJ). 2,3-Naphthotriazole (NAT) was synthesized from DAN according to Wheeler et al. [30].

2.2. NAT decomposition kinetics

In the present study, all kinetic experiments were conducted in a fluorometric microplate reader (Infinite M200, Tecan Austria GmbH) with a 96-well microplate format (Nunc, Denmark) at 37 °C. The fluorescence intensity of NAT was measured at excitation and emission wavelengths of 380 and 460 nm (gain = 93), respectively. The reaction buffer consists of 10 mM phosphate buffer (pH 7.4), DTPA (0.1 mM), and catalase (120 U/ml). A typical kinetic run was carried out in a reaction solution (final volume 300 μL) containing 0.5 μM NAT (25 μM stock solution in DMSO), with or without nitroxidative agents. Kinetic runs were initiated by adding various nitroxidative agents alone or in combination to the reaction solution and the fluorescence intensity was followed at 10-min or 5-min intervals up to 120 min. The specific information regarding the preparation of nitroxidative agents in each experimental setup is as follows: (1) the SPENO/SOTS-1 system. The stock solutions for SPENO and SOTS-1 were prepared in 0.01 M NaOH and DMSO, respectively. The final SOTS-1 concentrations in the reaction solution were fixed at 200, 400, 600 μM and the concentrations of SPENO co-added were in the range of 1–200 μM, 60–450 μM, and 60–600 μM, respectively. (2) The peroxynitrite system. An aliquot of 6 μL of diluted peroxynitrite stock solutions (in 0.3 M NaOH) was added to the reaction buffer, giving a final concentration of 50, 100, 200, 300, and 400 μM.

2.3. Estimation of NO fluxes

A spectrophotometric method was used to determine the rate of NO release from SPENO in the reaction system. Specifically, the decomposition kinetics of SPENO (180 μM) in the reaction buffer was determined by measuring the absorbance decrease at 252 nm over time (37 °C) in a UV–visible spectrophotometer (Shimadzu UV-2450, Kyoto, Japan). The first-order rate constant (k_1) for SPENO decomposition was obtained by fitting the kinetic trace. In the present study, the value for k_1 was estimated to be $4.5 \times 10^{-3}\ min^{-1}$. Since the decomposition of 1 mol of SPENO yields 2 mol of NO [31], the rate of NO release at any time t can be estimated from the following equation:

$$\frac{d[NO]}{dt} = 2k_1 \cdot [SPENO]_0 \cdot e^{-k_1 \cdot t} \quad (1)$$

where $[SPENO]_0$ is the initial concentration of SPENO. Thus, the initial NO flux can be calculated from Eq. (2):

$$\frac{d[NO]}{dt} = 2k_1 \cdot [SPENO]_0 \quad (2)$$

2.4. Estimation of superoxide fluxes

The rate of superoxide release from SOTS-1 was determined using a cytochrome c reduction assay. Briefly, cytochrome c was added to the same reaction buffer mentioned above to give a final concentration of 40 μM. To start a kinetic run, SOTS-1 (15–120 μM) was added to the cytochrome c containing buffer. The increase of absorbance at 550 nm as a result of cytochrome c reduction was then followed for 20 min (37 °C). It has been shown that 1 mol of SOTS-1 releases 0.4 mol of superoxide and the process follows first-order kinetics (Eq. (3), [32]):

$$\text{SOTS-1} \xrightarrow{k_2} 0.4O_2^{\cdot-} \quad (3)$$

Eq. (4) shows the reduction of cytochrome c (Fe^{III}cytc) by superoxide; accordingly, the formation of reduced cytochrome c (Fe^{II}cytc) is first-order with respect to superoxide and first-order with respect to oxidized cytochrome c, giving overall a second-order rate expression (Eq. (5)). Under a pseudo-steady-state condition, the rate of cytochrome c reduction is approximately equal to the rate of superoxide production, which is first-order with respect to SOTS-1 (Eq. (5)):

$$O_2^{\cdot-} + Fe^{III}\text{cytc} \xrightarrow{k_3} Fe^{II}\text{cytc} \quad (4)$$