Reaction Kinetics for Nitrosation of DAF-2 in Air Saturated Nitric Oxide Solution

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Abstract: Understanding the reaction rate constant of a probe compound with its target molecule is essential for its selection and use in biological and non-biological systems. Over the past decade, the probe, 4, 5-diaminofluorescein (DAF-2) has been widely employed for the detection and imaging of nitric oxide (NO•) in various media. However, the rate constant for the nitrosation of DAF-2 in air-saturated nitric oxide solution is far from being understood. For the first time, we have determined the second order rate constant for the reaction of DAF-2 with NO• (k_2) in air-saturated solution using competition kinetics method. An alternative competition kinetics method which involves a reciprocal plot of the reactant (DAF-2) and triazolofluorescein (DAF-2T), the product formed from the reaction of DAF-2 with NO• was developed and compared to the standard competition kinetics method. Our particular approach in this system is based on the use of oxy-hemoglobin (HbO_2), a potent scavenger of NO• against the DAF-2 which serves as the detector molecule. The product, DAF-2T, is separated from the reaction mixture by reversed-phase high performance liquid chromatography and its fluorescence intensity signals were measured at excitation and emission of 495 and 515 nm respectively. The results showed that the second order reaction rate constant of DAF-2 with NO• in air-saturated aqueous solution are comparable, with the average value of $(6.28 \pm 0.45) \times 10^6$ $M^{-1}s^{-1}$. Also, DAF-2 can react with NO• directly thereby by-passing the N_2O_3-mediated formation of DAF-2T at low NO• formation rates.

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1. Introduction

Fluorescein is widely used in biology as a fluorophore because of its convenient absorption wavelength for biological measurement, high extinction coefficient and high fluorescence quantum yield in water (Katayama et al., 1998; Kojima et al., 1998 a, 1998 b; Kojima and Nagano, 2000; Nagano, 1999). Over the past decade, DAF-2 has been extensively used as a chemical probe for the detection and imaging of NO• generated under physiological conditions or nitrite ion (NO_2^-) under acidic conditions conditions (Leikert et al., 2001; Nakatsubo et al.,1998 ; Planchet and Kaiser, 2006; Rathel et al., 2003; Suzuki et al., 2002). In fact, it has been reported that diaminofluoresceins (DAFs) have been applied to the determination of NO• in more than 100 scientific reports in the last 2 years (Gomes et al., 2006). Recently, we reported for the first time the application of this probe to the measurement of photochemically generated NO• in natural waters (Olasehinde et al., 2009; Olasehinde et al., 2010). However, despite the compelling evidence that DAF-2 can be used for the sensitive analysis of NO• in various media, the reaction rate constant of this probe with NO• has always been a puzzle. It should be pointed out that the reactive oxygen species along with other free radicals can react with NO• in competition with reaction with the probe (Olasehinde et al., 2009; Olasehinde et al., 2010; Zafiriou and Mc Farland, 1980). Therefore, information on the reaction rate constant of DAF-2 with NO• in air-saturated solution is critical to assessing the competition quantitatively, comparing the products of rate constant and the concentration. This will ultimately unravel the potency of DAF-2 for nitric oxide assay.

Pulse radiolysis is a direct method and is known to be the best for rate constant determinations (Ashton et al., 1995; Joseph and Aravindakumar, 2000). However, its use is often limited by its expensive nature and hence is not available to many radical chemists. Therefore, the competition kinetics method is employed for rate constant determinations (Gholami et al 1998; Kochany and Bolton, 1991; Manoj and Aravindakumar, 2003; Onstein et al.,1999). On this basis, we have carried out a series of experiments to determine second order reaction rate constant of DAF-2 with NO• (k_2) in air-saturated aqueous solution using competition kinetics methods. In the competition assay, information on the reaction rate can be obtained when the chemical reaction between the target molecule and a potential scavenger is examined in the presence of a detector molecule.

In this study, the detector molecule is DAF-2 and HbO_2 serves as the scavenger. Nitric oxide was generated *in situ* according to the equation (1) by the irradiation of NO_2^- with simulated light in air-saturated aqueous solution, which reacted competitively with the scavenger oxy-hemoglobin (HbO_2) and the detector molecule (DAF-2). It is well known that all amine-based probes such as DAF-2 are unreactive toward NO• without initial oxidation (Jourd'heuil, 2002; Espey et al., 2002; Williams, 1997; Wardman, 2007). Jourd'heuil (2002) suggested that •OH and $NO_2^{\bullet}$ are capable of rapid reaction with DAF-2 to generate DAF-2 radical intermediate, which can then react with NO• to form DAF-2T. As pointed out by Wardman (2007), DAF-2 radical intermediate can also be generated from the photoexcitation of DAF-2 probe. In our system, a possible mechanism for the formation of DAF-2T involves a one-electron oxidation of DAF-2 via the reaction of DAF-2 with free radicals (such as hydroxyl radicals) or/and photosensitized oxidation of DAF-2 to a free radical derivative, which reacts with NO• via radical-radical combination (eqs 2 and 3; Williams, 1997; Wardman, 2007).

$$NO_2^- + H_2O + h\nu \xrightarrow{k_1} NO\bullet + \bullet OH + HO^- \quad (1)$$

$$DAF\text{-}2 \xrightarrow{-e^-} DAF\text{-}2^{+\bullet} + H^+ \quad (2)$$

$$DAF\text{-}2^{+\bullet} + NO\bullet \xrightarrow{k_2} DAF\text{-}2T + H_2O \quad (3)$$

$$HbO_2 + NO\bullet \xrightarrow{k_3} metHb + NO_3^- \quad (4)$$

The product, DAF-2T, which is formed in the reaction between NO• and DAF-2 (Figure 1) in air-saturated solution is quantified by reversed-phase HPLC equipped with a fluorescence detector. The decrease in the amount of DAF-2T that was formed due to the presence of HbO_2 depends on the initial concentrations of DAF-2 and HbO_2, the concentration of NO• generated from NO_2^- and the ratio of the rate constants of (3) and (4). Since the reaction rate constant of HbO_2 with NO• is known, the value for (k_2) was determined using different ratios of HbO_2 and DAF-2.

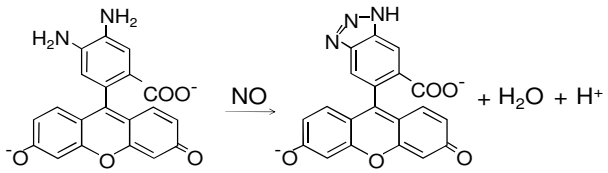

Fig.1 Structures of DAF-2 and DAF-2T

2. Material and Methods

2.1. Reagents and Chemicals

All reagents were reagent grade and used as received unless otherwise stated. All solutions were prepared with ultra-pure water obtained from a Milli-Q Plus system (Millipore; ≥ 18.2 MΩ cm). Acetonitrile and benzene (HPLC grade, > 99.5), and disodium hydrogen phosphate heptahydrate (guaranteed reagent) were obtained from Nacalai Tesque, (Tokyo, Japan). Sodium dithionite, sodium azide, bovine hemoglobin and sodium phosphate monobasic monohydrate were obtained from Sigma - Aldrich Japan. DAF-2 was obtained from Daiichi Pure Chemicals (Japan) and DAF-2T from Alex Biochemicals (Japan). NO_2^- standard solution (1000 mg L^{-1}) was obtained from Kanto Chemical Co. Inc.

2.2. Equipment

An HPLC system consisting of a PU-2089 plus pump (Jasco, Japan), a Rheodyne injection valve (Cotati, CA, USA) with a 100µL sample loop and a FP-2020 plus intelligent fluorescence detector (Jasco, Japan) interfaced with a C-R6A Chromatopac integrator (Shimadzu, Japan) was used. The separations were carried out on a RP-18 GP column (150 × 4.6mm I.D.,5µm) from Kanto Kagaku (Japan) with 10 mM phosphate buffer saline (PBS) at pH 7.4 with 6% acetonitrile as eluent at a flow rate of 1 mL min^{-1}. The detector was operated at 495nm and 515 nm for excitation and emission respectively.

2.3. Purification of HbO_2

To prepare HbO_2 from the commercial bovine hemoglobin, 1 mM sodium dithionite was added to a solution of bovine hemoglobin in 100 mM potassium phosphate at pH 7.0 to reduce any met-hemoglobin present in the solution. The mixture was dialyzed overnight at 4°C in 100 mM potassium phosphate, pH 7.0, to remove the residual dithionite. The concentration was determined by the previously reported method (Herold and Rock**,** 2003).

2.4. Irradiation Experiment

A solar simulator (Oriel model 81160-1000, Oriel corp.) equipped with a 300 W Xe lamp (ozone free, model 6258, Oriel corp.) was employed for the irradiation experiments. To simulate actual solar irradiance, wavelengths less than 300 nm were filtered out by optical filters (Oriel AM 0 and AM1.0, Oriel Instruments). The daily actinic flux of the solar simulator was determined by chemical actinometry (2-Nitrobenzaldehyde, 2-NB) (Arakaki et al., 1999). The photochemical reactions were performed in a custom-made quartz cuvette cell (Workshop for advanced Techniques, Hiroshima University). In this system, samples were illuminated in a 7-mL quartz cuvette cell. The solution inside the cell was gently stirred with a Teflon stirring bar and maintained at ca. 20°C using a Neslab RTE 111 recirculatory water bath. Aliquots of the irradiated

samples were removed at suitable intervals for HPLC analysis.

3. Results and Discussion

3.1 System Validation

3.1.1 Degradation of DAF-2 and Formation of DAF-2T

In our previous study (Olasehinde et al., 2009), we reported the selectivity towards NO• of the reaction transforming DAF-2 into DAF-2T. It was shown that the signal intensity of DAF-2T increased with an increase in the NO_2^- concentration. This suggests that DAF-2 reacts with the photoformed NO• generated from NO_2^- photolysis to produce DAF-2T in a concentration-dependent manner.

In this study, we assumed that DAF-2 concentration remained essentially constant during the course of the reaction and that DAF-2T formation was negligible. These assumptions were checked by irradiating 5 mL of Milli-Q water containing 5 μM DAF-2 for 30 min. An aliquot of the reaction mixture was subjected to HPLC analysis before and after the illumination. Concentrations of DAF-2 and DAF-2T were determined using calibration standards prepared in phosphate buffer (0.1 M, pH 7.4) and run on the day of the illumination experiment. The analysis of these samples (n =3) by HPLC showed a < 2% reduction in the concentration of each DAF-2. Meanwhile, DAF-2T was detectable in small amounts, but the concentration was always similar in the pairs of samples. As reported by Olasehinde et al (2009), DAF-2T was present as an impurity in the DAF-2, as opposed to being formed during the reaction period. We and others have also shown that stable oxidized forms of NO•, such as NO_2^- and NO_3^-, and other reactive oxygen species, such as •OH, O_2•$^-$, H_2O_2, and $ONOO^-$, do not react with DAF-2 to yield any fluorescent product and that DAF-2T is not formed in the absence of NO• (Gomes et al., 2006; Olasehinde et al., 2009; Kojima et al., 1998 b). This clearly indicates that the increase in fluorescence intensity of DAF-2T was dependent on the NO• concentration rather than the OH radicals in equation (1).

However, it is a well-known fact that the reactions of •OH and NO• are diffusion-controlled (Mack and Bolton, 1999) with a second-order rate constant of 1.0×10^{10} $M^{-1}s^{-1}$. To examine the scavenging effects of OH radicals on NO• in equation 1, a 5 mL Milli-Q water containing 10 μM NO_2^- was irradiated with 5 μM DAF-2 in the absence and presence of 1.2 mM benzene. We experimentally confirmed that 1.2 mM benzene is sufficient to effectively scavenge all •OH formed from the irradiation of 10 μM NO_2^- in Milli-Q water (data not shown), which agrees with the conclusion of the previous investigator (Nakatani et al., 2007). If the scavenging effect of •OH on the photoformed NO• were not negligible, the fluorescence intensity of DAF-2T would be expected to increase significantly upon the addition of •OH scavenger to compensate for the loss of NO• due to its reaction with •OH.

Contrarily, the result showed no appreciable difference between the fluorescence intensity of DAF-2T formed in the presence and absence of benzene (Figure. 2a), suggesting the negligible effect of OH radicals on the nitric oxide generated in equation 1. Further, it has been shown that 2 μM DAF-2 was sufficient to effectively scavenge all NO• formed from the irradiation of 10 μM NO_2^- in Milli-Q water in the presence of other *in situ* generated radicals (Olasehinde et al., 2009). In this study, 5 μM DAF-2 was employed to scavenge all NO• formed in the presence of other *in situ* generated scavengers.

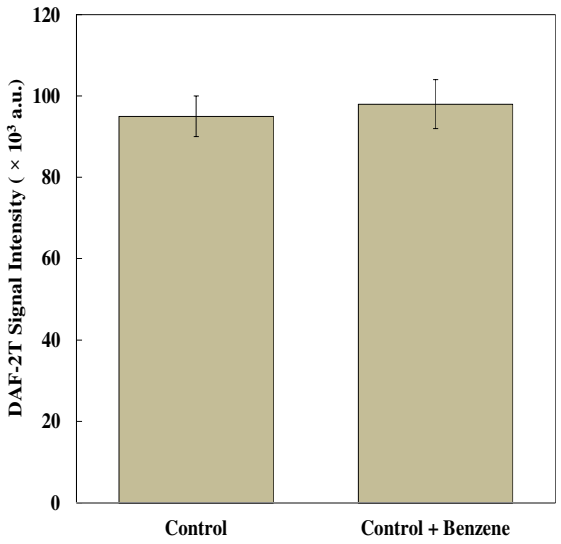

Figure 2a: The signal intensity of DAF-2T produced by the irradiation of 10 µM NO_2^- and 5 µM DAF-2 for 20 min in air-saturated solution (control) and with the addition of 1.2 mM benzene. Each experiment was conducted in triplicate.

If we assume that the steady-state concentration of •OH in equation 1 is as high as ~ 10^{-15} M (a reasonable upper limit based on the •OH photoformation rates from NO_2^- photolysis and the scavenging rate constant of •OH by NO_2^-, ignoring loss of •OH due to other mechanisms) and the second order rate constant for the recombination reaction of the NO• and OH radicals of 1.0×10^{10} $M^{-1}s^{-1}$ (Mack and Bolton, 1999; Buxton et al., 1988), then equation 5 proceeds with a first–order rate constant of 1.0×10^{-5} s^{-1}.

$$\text{•OH} + \text{NO•} \xrightarrow{k_4} \text{HNO}_2 \qquad (5)$$

In contrast, if k_2 is 6.28 × 10^6 M^{-1} s^{-1} and the DAF-2 concentration is 5 μM, equation (3) has a first-order rate constant of 31 s^{-1}, a 6-order of magnitude higher than equation (5). From this simple analogy, it is obvious that the effect of •OH on NO• in equation 1 is negligible at the optimum DAF-2 concentration employed in this study.

3.1.2 Specificity of DAF-2 for NO

The nitrosating species in the reaction of DAF-2 with NO• in air-saturated solution has long been a subject of controversy. A number of studies have established that the nitrosating species in the reaction between DAF-2 and NO• in air-saturated solution is N_2O_3, suggesting that NO• does not nitrosate DAF -2 directly and that the nitrosation of DAF-2 in aerobic solution is consistent with the formation of N_2O_3 consequent to NO• autoxidation (Gomes et al., 2006; Nakastubo et al . 1998; Rathel et al., 2003; Nagano et al., 1995; Leikert et al., 2001, Kojima et al., 1998b; Nakastubo et al . 1998; Rathel et al., 2003; Nagano et al., 1995; Leikert et al., 2001, Kojima et al, 1998b).

$$2NO\bullet + O_2 \xrightarrow{k_5} 2NO_2 \quad (6)$$

$$NO\bullet + NO_2^- \underset{k_7}{\overset{k_6}{\rightleftharpoons}} N_2O_3 \quad (7)$$

$$DAF\text{-}2 + N_2O_3 \xrightarrow{k_8} DAF\text{-}2T + NO_2^- + H^+ \quad (8)$$

However, recent studies have shown that DAF-2T formation is not strictly related to the reaction with N_2O_3 (Espey et al, 2002; Jourd'heui et al., 2002). These authors hypothesized that the triazole product (DAF-2T) may be formed predominantly by oxidative nitrosylation, a process that involves oxidation of the probe followed by direct reaction with NO• (Espey et al., 2002). In a separate study using 2, 3-diaminonaphthalene (DAN), an analogue of DAF-2, for trapping NO• in seawater, the results showed that the fluorescence intensity between oxygenated and de-oxygenated DAN solutions was in the range of detection error, suggesting that DAN can react with NO• directly in aerobic solutions (Liu et al., 2009).

To better understand the contribution of N_2O_3 to DAF-2T fluorescence, we performed a series of inhibition experiments by irradiating 5 mL of Milli-Q water containing air-saturated solution of 10 μM NO_2^- and 5 μM DAF-2 in the presence and absence of N_2O_3 scavenger sodium azide. Although the rate constant for the reaction of DAF-2 with N_2O_3 (k_8) is not known, azide reacts with N_2O_3 at a diffusion controlled rate, with a second order reaction rate constant of 2 × 10^9 $M^{-1}s^{-1}$ (Williams, 1997). If we assume that k_8 is as high as ~ 10^9 $M^{-1}s^{-1}$, and that N_2O_3 were the intermediate responsible for the nitrosation of DAF-2 in air-saturated solution, equation 8 proceeds with a first-order rate constant of 5.0 × 10^3 s^{-1} at 5 μM DAF-2 employed in this study. However, using the published rate constant of N_2O_3 with azide (Williams, 1997). and at 0.5 mM [N_3^-], about 99.5 % of the observed DAF-2T should be quenched. In contrast, addition of 0.5 mM sodium azide showed no detectable decrease in the fluorescence intensity of DAF-2T (Figure 2b), a discrepancy which appears to preclude the mechanisms described by eqs 6-8 under our experimental conditions.

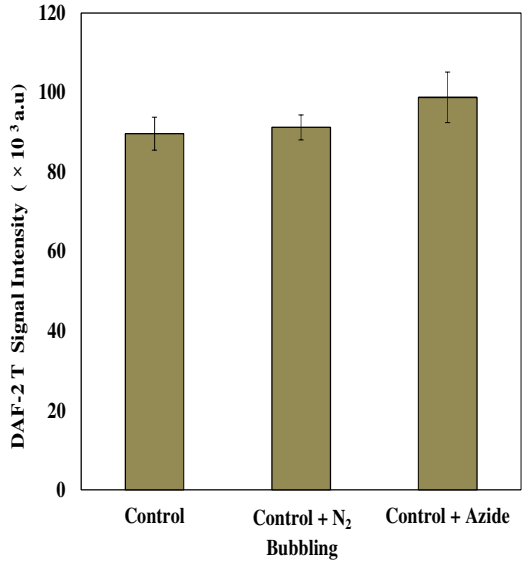

Figure 2b: The signal intensity of DAF-2T generated by the irradiation of 10 µM NO_2^- with 5 µM DAF-2 for 20 min in air-saturated solution (control), with the addition of 0.5 mM sodium azide, and when the solution was purged with N_2 for 30 min prior to irradiation. Each experiment was conducted in triplicate.

As shown in Figure 2c, when NO• scavenger HbO_2 (0.1- 0.4 μM) was added to the reaction mixture containing 10 μM NO_2^- with 5 μM DAF-2 followed by irradiation, a concentration-dependent reduction in the fluorescence intensity of DAF-2T was observed. Furthermore, the possibility of DAF-2T formation in anaerobic solution, bypassing the requirement for NO• autoxidation for the formation of DAF-2T was investigated. In each of these experiments, a 5-mL Milli-Q water containing 10 μM NO_2^- and 5 μM DAF-2 was purged with a high-purity N_2 gas for 30 min to remove oxygen prior to irradiation. The solutions were then irradiated for

20 min and analyzed for DAF-2T. The difference in the fluorescence intensity of DAF-2T in the aerobic solutions compared to the anaerobic solutions was < 5%, which is within the analytical error (See Figure 2b).

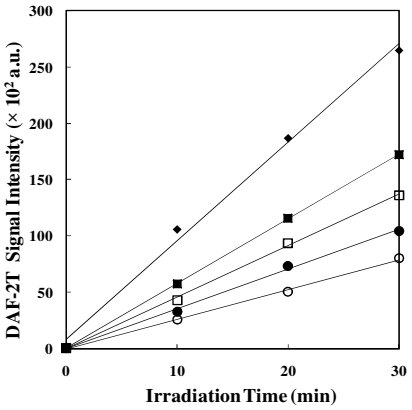

Figure 2c Signal intensities of DAF-2T produced by the irradiation of 10 μM NO_2^- and 0.5 μM DAF-2 in Milli-Q water with and without HbO_2 as a function of irradiation time. Closed diamonds: 0 µM HbO_2; closed rectangles: 0.1 µM HbO_2; open rectangles: 0.2 µM HbO_2; closed circles: 0.3 µM HbO_2; open circles: 0.4 µM HbO_2.

To further clarify the likelihood of equation 6 to generate N_2O_3 compared to the loss of NO• via equation 3, the first order rate constants for eqs 6 and 3 were estimated. If we assume that the steady-state concentration of NO• = ~ 10^{-15} M (based on the ratio of the NO• photo-formation rate from NO_2^- and the scavenging rate by NO_2^-, ignoring the loss of NO• due to other processes), k_6 = 1.1 × 10^9 $M^{-1}s^{-1}$ (Mack and Bolton, 1999), the forward half-reaction for equation 6 proceeds with a first-order rate constant of 1.1 × 10^{-6} s^{-1}. As mentioned earlier, equation 3 proceeds with a first-order rate constant of 31.1s^{-1} indicating a 7-order of magnitude higher than the equation 6.

Taken together, these data signify that the nitrosating species under our experimental conditions is NO• rather than the addition of N_2O_3. This is reasonable because previous reports (Espey et al., 2002; Wink et al., 1994), demonstrated that at low NO• formation rates (~ nM NO• / min), the half-life of NO in aerobic environments is potentially quite long, which may allow other pathways such as the reaction of NO• with oxy-hemoglobin (or other NO• probe compounds) to predominate over the nitrosonium addition by N_2O_3. This result suggests that DAF-2 can react with NO• directly (as in equation 3), thereby by-passing the N_2O_3-mediated formation of DAF-2T. However, nitrosation via NO• autoxidation may be the dominant route when relatively high rates of NO• formation occur under aerobic condition which would favour nitrosation through N_2O_3 formation subsequent to the reaction of NO_2 with NO• (Espey et al., 2002).

3.2 Determination of K_2 using Standard Competition Kinetics

We employed the standard competition kinetics method (Buxton et al., 1988) to determine the reaction rate constant of DAF-2 with NO• (k_2) in air-saturated aqueous solution. Nitric oxide radical was generated by irradiating 10 μM NO_2^- in a 7-mL quartz cell. DAF-2 reacts with NO• in air-saturated solution yielding the highly fluorescent DAF-2T. Also, HbO_2 reacts with NO• to produce methemoglobin (metHb), with a second order reaction rate of 3.7 × 10^7 M^{-1} s^{-1} (Gross and Lane, 1999). In experimental solution containing DAF-2 without any other NO• scavenger, all NO• formed reacts with DAF-2 to produce DAF-2T. The total amount of DAF-2T formed under this condition is denoted as Q_T. However, if HbO_2 is present in the experimental solution with DAF-2, both reactants compete for the NO• produced which leads to the attenuation of the signal intensity of DAF-2T compared to the reaction of DAF-2 with NO• in the absence of HbO_2. In this system, the amount of DAF-2T formed is a fraction of the total NO• generated which is denoted as DAF-2T '. If the concentration of DAF-2T ' is represented by Q and Q' represents the concentration of metHb, therefore the total scavengable NO• under this condition is given as:

$Q_T = Q + Q'$

$(dQ/dt) / (dQ'/dt) = (Q / Q')$ (9)

$(dQ/dt) = k_2 [NO•] [DAF\text{-}2]$

$(dQ'/dt) = k_3 [NO•] [HbO_2]$

$Q / Q' = (k_2 [NO•] [DAF\text{-}2]) / (k_3 [NO•] [HbO_2])$ (10)

Since Q is the measurable product and Q' is expressed in terms of Q

Therefore

$Q' = Q_T - Q$ (11)

Substitute eq 11 in eq 10

$Q / (Q_T - Q) = (k_2 [DAF\text{-}2]) / (k_3 [HbO_2])$ (12)

Inverse of eq 12 gives

$(Q_T - Q) / Q = (k_3 [HbO_2]) / (k_2 [DAF\text{-}2])$ (13)

eq 13 is simplified as

$\{Q_T / Q\} = 1 + \{(k_3 [HbO_2]) / (k_2 [DAF\text{-}2])\}$ (14)

Let

$Q_T = [DAF\text{-}2T]_o$, $Q = [DAF\text{-}2T]'$

Therefore, eq 14 becomes

$[DAF\text{-}2T]_o / [DAF\text{-}2T]' = 1 + \{ (k_3 [HbO_2]) / (k_2 [DAF\text{-}2]) \}$ (15)

In equation 15, [DAF-2T] ' and $[DAF\text{-}2T]_o$ are the concentrations of DAF-2T produced from the reaction of DAF-2 and NO• in air-saturated solutions with and without HbO_2 respectively, $[HbO_2]$ is the initial concentration of HbO_2, [DAF-2] is the initial

concentration of DAF-2, k_3 is the reaction rate constant of HbO_2 with NO• ($M^{-1}s^{-1}$) and k_2 is the reaction rate constant of DAF-2 with NO• ($M^{-1}s^{-1}$). We carried out a series of experiments by 30-min irradiation of 10 μM NO_2^- with (0.05 – 0.1 μM) HbO_2 varying the concentration of DAF-2 from (0.1 - 0.5 μM). When the DAF-2 concentration was held constant and the concentration of HbO_2 was increased, a concentration-dependent reduction in the amount of DAF-2T ' was observed. Contrarily, when the concentration of HbO_2 was held constant while increasing the concentration of DAF-2, the signal intensity of DAF-2T ' was enhanced to compensate for the increase in the concentration of DAF-2. However, to determine the concentration of the Q_T produced 10μM NO_2^- was irradiated with 5 μM DAF-2 in the absence of HbO_2 for 30 min. As reported earlier (Olasehinde et al., 2009), the concentration dependence of DAF-2 on NO• generated from the irradiation of 10 μM NO_2^- in Milli-Q water revealed that 5 μM DAF-2 is sufficient to effectively scavenge all NO• formed. A plot of $[DAF\text{-}2T]_o$ / [DAF-2T]' vs $[HbO_2]$ / [DAF-2] for separate experiments with solutions of varying $[HbO_2]$ / [DAF-2] ratio, based on eq 15 is shown in Figure 3. Thus, the value for k_2 estimated from the slope of this graph and eqn 15 was (6.17 ± 0.26) × 10^6 M^{-1} s^{-1} (mean ± standard deviation, n =3).

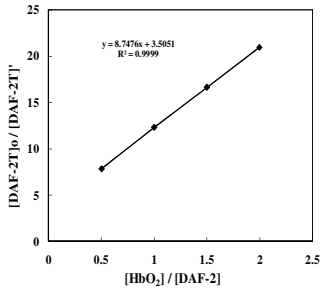

Figure 3: Graph for the calculation of the rate constant for the reaction of DAF-2 with NO radical using the competition kinetics method, correlation coefficient (r^2) = 0.999.

3.3. Determination of k_2 using Alternate Competition Kinetics Method

We proposed an alternate competition kinetics method for the determination of reaction rate constant of DAF-2 with NO• to ensure that the result obtained from the standard competition kinetics method was not a mere artifact. The following mathematical derivation will aid in the determination of k_2.

Given that $R_{NO} = R_{DAF\text{-}2T}/(F_{DAF\text{-}2,\,NO})\,(Y_{DAF\text{-}2T})$ (16)

and

$F_{DAF,\,NO} = (k_2)\,[DAF\text{-}2] / \sum (ks_{,\,NO}\,[s])$ (17)

Where $R_{DAF\text{-}2T}$ is the formation rate of DAF-2T (M /s), $Y_{DAF\text{-}2T}$ is the yield of DAF-2T per DAF-2 oxidized by NO•, $F_{DAF\text{-}2,\,NO}$ is the fraction of DAF -2 that reacts with NO•, k_2 is the reaction rate constant of DAF-2 with NO• ($M^{-1}s^{-1}$), R_{NO} is the formation rate of NO• and $\sum(ks_{,\,NO}\,[s])$ is the summation of scavenging rate of NO• with NO• scavengers in experimental solution.

In an experimental solution containing DAF-2, HbO_2 and other *in situ* generated scavengers of NO•, the summation of scavenging rate of NO• by the scavengers is given in Eq. 18.

$\sum (ks_{,\,NO}\,[s]) = (k_2)\,[DAF\text{-}2] + k_3\,[HbO_2] + k_a\,[S_a] + k_b\,[S_b] + \ldots\ldots + k_n\,[S_n]$ (18)

where $k_a \ldots\ldots k_n$ are the rate constants of individual scavengers $S_a \ldots S_n$ with NO•

However, If HbO_2 and DAF-2 are the main scavengers of NO• in the experimental solution such that the total NO• consumption excluding HbO_2 and DAF-2 is significantly lower than HbO_2 and DAF- 2, that is: $\{ (k_2)\,[DAF\text{-}2] + k_3\,[Hb] \} >>>> \sum (k_a[S_a] + k_b[S_b] + \ldots\ldots\ldots\ldots + k_n\,[S_n]$,

Then

$\sum(ks_{,\,NO}\,[s]) = (k_2)[DAF\text{-}2] + k_3[HbO_2]$ (19)

Substituting Eq. (19) in Eq. (17) gives

$F_{DAF\text{-}2,\,NO} = k_2[DAF\text{-}2] / \{k_2\,[DAF\text{-}2]+k_3[HbO_2]\}$ (20)

Substituting Eq. (20) in Eq. (16) and re-arranged gives

$R_{DAF\text{-}2T} = R_{NO}\{ (k_2\,[DAF\text{-}2])(Y_{DAF\text{-}2T}) \} /\{ k_2\,[DAF\text{-}2] + k_3[HbO_2]\}$ (21)

This can then be solved algebraically in terms of $1/R_{DAF\text{-}2T}$ to give:

$1/ R_{DAF\text{-}2T} = \{k_2\,[DAF\text{-}2] + k_3[HbO_2] \} / R_{NO}\{ (k_2\,[DAF\text{-}2]) (Y_{DAF\text{-}2T}) \}$ (22)

Simplifying Eq. (22) gives

$1/R_{DAF\text{-}2T} = k_3\,[HbO_2] / R_{NO}\{ (k_2\,[DAF\text{-}2]) (Y_{DAF\text{-}2T}) \} + 1/ R_{NO}\,(Y_{DAF\text{-}2T})$ (23)

Hence, a plot of $1/R_{DAF\text{-}2T}$ versus 1/ [DAF-2] will give a line with

slope = $k_{3\,,}\,[Hb] \times$ y- intercept / k_2 (24)

Using this methodology, we performed a series of independent experiments by irradiating (5 -10 µM) NO_2^- in Milli-Q water with various concentration of DAF-2 (0.1–1 µM) in the presence of (0.1 – 0.3 µM) HbO_2. For each experiment, NO_2^- was irradiated with a fixed concentration of HbO_2 while varying the concentration of DAF-2 used. Aliquot of the irradiated sample was withdrawn for analysis at suitable intervals and the concentration of DAF-2T produced was determined. Photo-formation rate of DAF-2T ($R_{DAF\text{-}2T}$) was calculated from the relationship between the cumulative irradiation time and the concentration of DAF-2T. From the relationship between $1/R_{DAF\text{-}2T}$ and 1/DAF-2 (Figure 4) using Eq. (24), the second order reaction rate constant of DAF-2 with NO• was estimated to be (6.39 ± 0.31) × 10^6 M^{-1} s^{-1} (mean ± standard deviation, n =3).

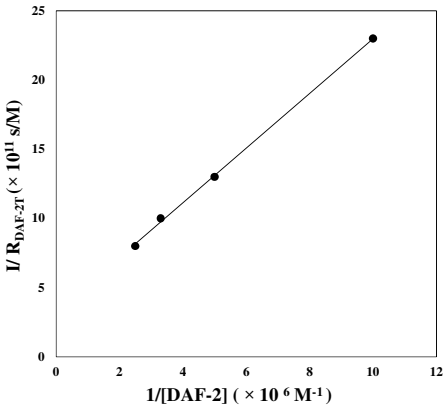

Figure 4. Graph for the calculation of the rate constant for the reaction of DAF-2 with NO• using the alternate competition kinetics method. A plot of reciprocal of DAF-2T photo-formation rate (1/ R_{DAF-2T}) and inverse of the concentration of added DAF-2 (1/[DAF-2]) in Milli-Q water. [NO_2^-]:10 μM , [DAF-2]: (0.1- 0.4 μM); [Hb] : 0.1 μM. The values for the y-intercept and slope are 3.45 × 10^{11} s/M and 1.93 × 10^5s respectively, correlation coefficient (r^2) = 0.993.

4. Conclusions

The second order reaction rate constant of DAF-2 with NO• has been determined. Using the two different competition kinetics approaches, reaction rate constants of DAF-2 with NO• were comparable, with the average value of (6.28 ± 0.45) ×10^6 $M^{-1}s^{-1}$. The strong agreement between the two competition kinetics procedures implies that the reaction between DAF-2 and NO• is considerably high. This study also reveals that at low NO• formation rates (~ nM NO• / min), DAF-2 can react with NO• directly thereby by-passing the N_2O_3-mediated formation of DAF-2T. From the results presented in this study, DAF-2 could serve as a novel probe compound for the sensitive determination of NO^- in biological samples as well as in aqueous solution.

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