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The kinetics of the reaction between NO and O_2 as studied by a novel approach

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Abstract

The kinetics of the reaction between NO and O_2 was determined by measuring the time course of the decrease in the concentration of NO with a quench–flow technique. NO and O_2 were mixed rapidly and reacted for periods of time varying from 10 to 50 s. A second rapid mixing with a solution containing an excess of deoxyhemoglobin and sodium hydrosulfite trapped free NO as nitrosylhemoglobin and reduced O_2. The spectrum of the mixture of deoxy- and nitrosylhemoglobin was recorded within 30 s from the second mixing, before any appreciable dissociation of NO from the protein, by means of a flow-cell mounted on-line with the quench–flow apparatus. The amount of NO not consumed in the auto-oxidation reaction was calculated from the proportion of nitrosylhemoglobin in the mixture. As NO and O_2 bind deoxyhemoglobin at comparable rates and NO is oxidized to nitrate by oxyhemoglobin, the ratio of hemoglobin/(NO + O_2) had to be optimized to avoid the interference of this oxidation reaction. The kinetics was first and second order with respect to O_2 and NO, respectively and third order overall with a rate constant $k = 4 \times k_{aq} = 4 \times 2.23\ (\pm 0.26) \times 10^6\ M^{-2}\ s^{-1}$ at 20°C, invariant in the pH range 7–9, in agreement with published values obtained by different methodologies. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Nitric oxide (NO) is the product of arginine metabolism and plays an important physiological role in neurotransmission, vasodilation, platelet aggregation and macrophage function [1,2]. Specific targets have been identified, such as the heme moiety of guanylyl cyclase [1,3], but a variety of other molecules, proteins and low molecular weight substances can also react with this radical. Some reactions yield products possibly implicated in physiological functions, e.g. as mediators of nitric oxide delivery to target molecules [4–7]. In other cases NO toxicity is used to inactivate

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molecules potentially harmful to the organism [8]. Oxygen dissolved in tissues reacts with NO, acting as a scavenger which prevents NO from reaching its targets [9,10]. Thus the auto-oxidation of NO has been studied to assess to what extent physiological concentrations of oxygen can influence the cellular functions of NO.

The mechanism of auto-oxidation of NO in the gas phase yields species NO_2 according to a third order rate law, although two bimolecular reactions may be involved [11]:

$$NO + O_2 \leftrightarrow OONO \quad (1)$$

$$OONO + NO \leftrightarrow ONOONO \rightarrow 2NO_2 \quad (2)$$

The product of NO_2 dimerization, O_2NNO_2, disproportionates in water yielding nitrous and nitric acids:

$$O_2NNO_2 + H_2O \rightarrow 2H^+ + NO_2^- + NO_3^- \quad (3)$$

The auto-oxidation of NO in the aqueous phase yields only nitrous acid, the overall stoichiometry of the reaction being:

$$4NO + O_2 + 2H_2O \leftrightarrow 4NO_2^- + 4H^+ \quad (4)$$

The mechanism of Reaction (4) has been studied by measuring either the increase in the concentrations of the products, NO_2^- and H^+ [9,10,12,13], or the decrease in the concentration of NO [14]. The first approach has yielded a rate law that is first and second order with respect to O_2 and NO, respectively, suggestive of a mechanism involving species N_2O_3 either directly, as in Reaction (5):

$$NO_2 + NO \leftrightarrow N_2O_3 \quad (5)$$

$$N_2O_3 + H_2O \leftrightarrow 2NO_2^- + 2H^+ \quad (6)$$

or through some other highly reactive unidentified intermediate [9]. In both cases the reaction involving NO_2 should not be rate limiting.

The determination of the change in NO concentration by means of a porphyrinic sensor has indicated a rate law of zero and first order with respect to NO and O_2, respectively, suggestive of a multi-step mechanism in which the reactions involving NO are not rate limiting [14].

Since the discrepancy between the two experimental approaches could be either methodological or theoretical we have used an alternative method for the measurement of the concentration of reacting NO to test the mechanism in aqueous medium. An aqueous solution of NO was mixed with an aqueous solution of O_2. After varying time periods the reactants were mixed with a quenching solution of deoxyhemoglobin (Hb) containing sodium hydrosulfite. The association reactions of NO and O_2 with hemoglobin are both rapid, but whereas the ligand dissociation from nitrosylhemoglobin (HbNO) is slow, it is orders of magnitude faster from oxyhemoglobin (HbO_2) [15]. Hydrosulfite, which reacts with free O_2 and NO, can remove all the O_2 from the quenched solution before any appreciable dissociation of NO from HbNO. The amount of NO not reacted with O_2 in the auto-oxidation reaction is then determined from the spectrum of the mixture of Hb and HbNO. Since several additional reactions involving NO and O_2 can occur after mixing the reactants with the hemoglobin/hydrosulfite solution, a thorough evaluation of the conditions and a number of controls were made to check that the procedure is a valid technique for quenching the auto-oxidation reaction of NO.

2. Experimental procedures

2.1. Materials

Stock solutions of NO were prepared by bubbling a mixture of NO and N_2 of a known composition in a carefully deoxygenated buffer. The gas mixture was purged through 2-M NaOH to remove traces of NO_2. Calibrated mixtures containing 6–15% (v/v) NO were purchased from SAPIO srl, Bergamo.

Ghost-free hemoglobin was prepared by centrifugation of red blood cell lysates and was equilibrated in 0.1 M KCl by gel filtration. Samples, 200-μl, were stored in liquid nitrogen at a concentration of 10 g/dl. The Hb solutions for the quenching experiments were prepared by diluting these samples in 50 mM sodium phosphate, pH 7,

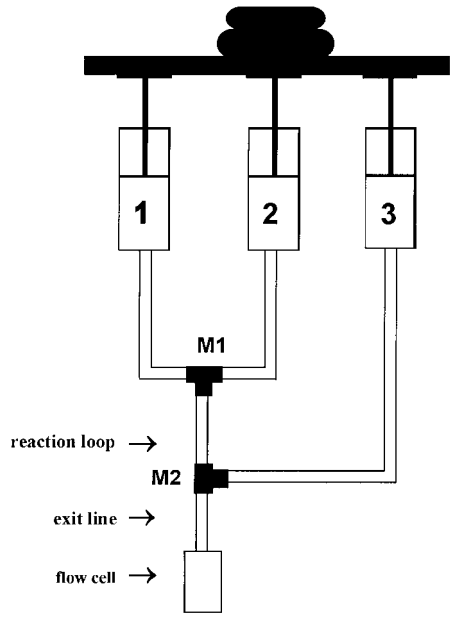

Fig. 1. Scheme of the quench–flow apparatus. 1 and 2, reactant syringes; 3, quencher syringe; M1 and M2, mixers. The reaction occurs in the reaction loop. The time spent by the solution in the exit line between M2 and the optical observation in the flow cell varied from 400 to 800 ms depending on the flow conditions.

and deoxygenated by scrubbing with N_2. The μM concentration of hemoglobin in this work is the heme concentration.

2.2. Experimental procedure

Fig. 1 shows a scheme of the quench–flow apparatus (KinTec RQF-3, University Park, PA). Syringes 1 and 2 were filled with the reactants, i.e. aqueous solutions of NO and O_2, respectively. Syringe 3 contained a solution of human hemoglobin, to trap NO not reacted with O_2, and sodium hydrosulfite, to remove O_2 not reacted with NO. The contents of syringes 1 and 2 were mixed in M1 and aged in the 'reaction loop' for a programed time (10 ms–50 s or longer). The reactants were then mixed in M2 with the contents of syringe 3. The liquid from M2 was analyzed by means of a flow-cell (1-cm optical path) connected to M2 by a teflon tube (the 'exit line' in Fig. 1) and mounted in a spectrophotometer (Beckman DU 70).

The apparatus was operated to yield turbulent flow under all conditions. Reaction times up to 700 ms were obtained by suitable adjustments of the flow and of the inner volume of the interchangeable reaction loops. For longer reaction times the quench–flow apparatus was operated in a push–push mode. The syringes, mixers and reaction loop were thermostatted at 20°C. Further aging of the liquid in the tube (the exit line in Fig. 1) between the mixing with the hemoglobin/hydrosulfite solution and the optical observation varied from 400 to 800 ms depending on the flow conditions. Spectra were recorded within 30 s after stopping the flow.

Since it was observed that the some of the materials of the original KinTec apparatus (tubes, connectors, etc.) interacted with NO, a new thermostatted reaction block was built using gas-tight Hamilton syringes and valves, stainless steel tubes and ball-mixers [16].

2.3. Spectral analyses

The concentration of NO that was not consumed in the auto-oxidation reaction was calculated from the proportion of HbNO in the mixture of HbNO/Hb. Spectra were recorded at 2-nm intervals in the 450–650-nm range. The experimental spectra were fitted with linear combinations of the molar spectra of Hb and HbNO. The Hb spectrum was measured using a protein solution deoxygenated by nitrogen tonometry prior to the addition of a trace amount of hydrosulfite. The spectrum was then normalized using the extinction coefficient value of 13.35 mM^{-1} cm^{-1} at 554 nm [17]. The extinction coefficient of HbNO was calculated from the spectrum of the same solution of Hb saturated with NO. The least squares unconstrained fit to the experimental spectra and the error estimation were carried out using Matlab 4.0 (MathWorks, Natick, MA, USA). Fig. 2 shows the agreement between an experimental spectrum and the fitted linear combination of the HbNO and Hb spectra. The error in the NO concentration, as determined by this procedure, was usually within 1%.

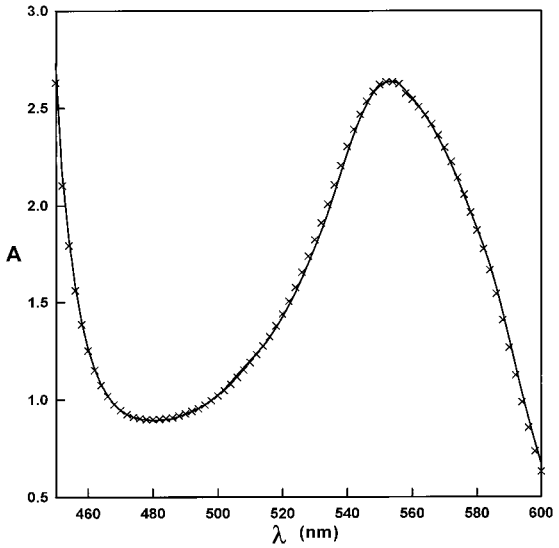

Fig. 2. (X), experimental spectrum of the solution observed in the flow cell after quenching the auto-oxidation reaction of NO with a Hb/hydrosulfite mixture. The spectrum was recorded within 30 s after stopping the flow. (———), fit to the experimental spectrum of a linear combination of the spectra of Hb and HbNO. $[HbNO]_{eme} = 42\ \mu M$, $[Hb]_{eme} = 157\ \mu M$. Similar spectra were obtained in the simulation experiments reported in Fig. 6, in which the Hb/HbNO mixture containing hydrosulfite was exposed to O_2, O_2^- and H_2O_2.

2.4. Kinetic simulations

Simulations of the binding kinetics of NO and O_2 to Hb were carried out assuming four sequential reactions for the association and dissociation of the two ligands acting independently (Fig. 3). This basic scheme was then modified to introduce additional reactions, such as the reaction of NO with HbO_2. A system of rate equations was developed according to this scheme, which was solved numerically using Matlab 4.0 programs and published values of the rate constants.

2.5. Validation of the procedure

Quenching of the auto-oxidation reaction requires that all NO not reacted with O_2 be trapped quantitatively as HbNO upon mixing the reactants with the solution of Hb/hydrosulfite in M2 (Fig. 1). The relevant reactions are:

$$NO + Hb \leftrightarrow HbNO \quad (7)$$

$$O_2 + Hb \leftrightarrow HbO_2 \quad (8)$$

$$O_2 + S_2O_4^{2-} \rightarrow (\text{products: } O_2^-, H_2O_2) \quad (9)$$

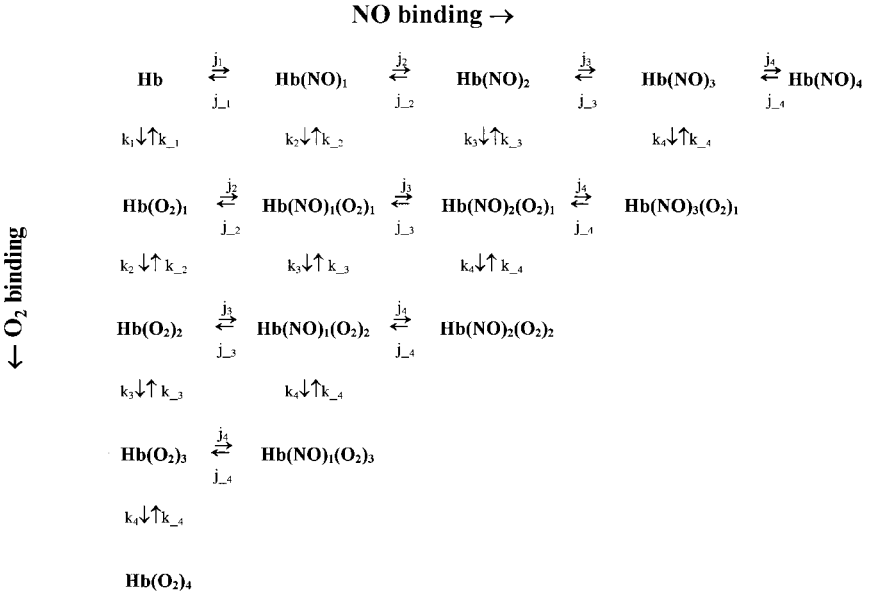

Fig. 3. Scheme of sequential independent binding of NO and O_2 to Hb used for the development of the system of rate equations for binding.

The quantitative determination of HbNO can be impaired by the occurrence of other reactions involving NO and O_2, which are listed in the following:

$$NO + S_2O_4^{2-} \rightarrow (\text{products}) \quad (10)$$

$$HbO_2 + NO \leftrightarrow Hb^+ + NO_3^- \quad (11)$$

$$HbNO + 1/2O_2 \rightarrow Hb^+ + NO_2^- \quad (12)$$

$$2NO_2^- + S_2O_4^{2-} \rightarrow 2NO + 2SO_3^{2-} \quad (13)$$

Specific controls or theoretical and, whenever possible, experimental simulations of the NO quenching procedure, described in the following, were carried out to check the effects of Reactions (10)–(13) on NO recovery as HbNO, which is crucial for the correct estimation of the rate of the auto-oxidation reaction.

2.5.1. Reaction (7)

The rate constant for the NO association to Hb is 24×10^6 $M^{-1}s^{-1}$ [18]. The NO dissociation reaction from nitrosylhemoglobin is 1.4×10^{-4} s^{-1} and approximately 100 times faster from the NO ligation intermediates [18,19]. A simulation of NO binding to Hb using these rate constant values (Fig. 4) indicated that under the conditions of the quenching experiments NO binds quantitatively to Hb within 3 ms and no appreciable NO dissociation occurs during the time (approx. 30 s from mixing in M2) required to record the hemoglobin spectra.

2.5.2. Reaction (8)

The rate constant for the O_2 association reaction of hemoglobin is in the 10^6–10^7 M^{-1} s^{-1} range, similar to that of NO [15]. The rate constant for the dissociation reaction is strongly dependent on the state of ligation of hemoglobin and is in the range 20–2000 s^{-1} [15]. A simulation of the NO binding kinetics in the presence of O_2 indicated that O_2 does not interfere with NO binding to Hb if the protein concentration provides enough vacant sites for the binding of both ligands, as also shown in Fig. 3. The simulation was carried out ignoring the effects of Reaction (11).

2.5.3. Reaction (9)

It is estimated that the rate of O_2 consumption is 3–5 $s^{-1} \times [S_2O_4^{2-}]$ at 20°C and neutral pH [15]. The rate of removal of O_2, which was not consumed by the auto-oxidation reaction of NO and was present in the quenched solution as free and hemoglobin-bound oxygen, depends on the hydrosulfite concentration because the dissociation of O_2 from oxyhemoglobin is too fast to be rate limiting. As the amount of NO trapped by Hb was calculated from the spectrum of a mixture of nitrosyl- and deoxyhemoglobin observed in the flow cell after stopping the flow, the oxygen removal had to be completed within the time (400–800 ms) elapsed after mixing in M2 and the flow cell. The concentration of hydrosulfite required for the complete removal of the various concentrations of O_2 used for the kinetic experi-

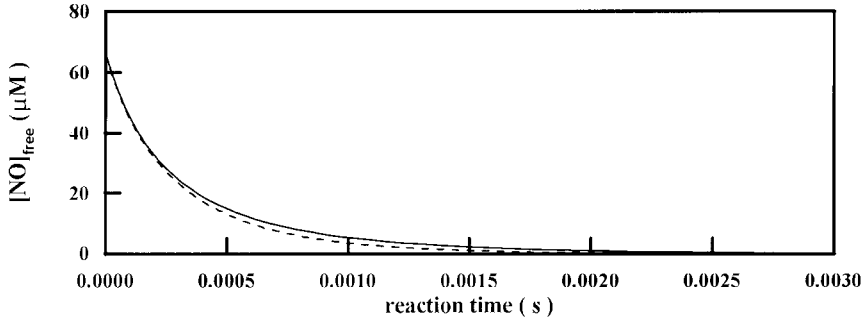

Fig. 4. Kinetic simulation of the binding of NO to Hb in the absence (- - - -) and in the presence (———) of O_2. The simulation was carried out assuming independent binding of the two ligands as shown in the scheme in Fig. 2 and ignoring the reaction between NO and HbO_2, which yields Hb^+ and nitrate. Other conditions: [NO] = 66 μM; [O_2] = 72 μM; [Hb] = 160 μM.

ments was determined as follows. Solutions of O_2 of varying titer contained in syringe 1 (Fig. 1) were mixed in M1 with the deoxygenated buffer contained in syringe 2 and then in M2 with quenching solutions of Hb containing varying concentrations of hydrosulfite. The formation of deoxyhemoglobin was monitored under conditions of continuous flow by measuring the absorbance change at 560 nm. The proportion of HbO_2 not reduced under the various conditions of hydrosulfite concentration and time of reaction was then calculated from the spectrum of the Hb/HbO_2 mixture in the 450–650-nm range recorded under continuous flow conditions. It was found that a 550-μM concentration of hydrosulfite was enough to completely reduce oxygen before the solution reached the flow cell under all experimental conditions.

2.5.4. Reaction (10)

The rate constant of the reaction of NO with hydrosulfite, Reaction (10), is 1.4×10^3 M^{-1} s^{-1} [18], approximately four orders of magnitude less than the rate constant of Reaction (7). To check that NO was quantitatively trapped by Hb in spite of the presence of a high concentration of hydrosulfite in the quenching solution, a stock solution of NO in 50 mM phosphate buffer, pH 7 and 20°C, was mixed in M1 with buffer, deoxygenated by nitrogen tonometry, and in M2 with solutions of Hb containing increasing amounts of hydrosulfite. The NO concentration values of the stock solution as measured in these control experiments are listed in Table 1.

Table 1
Concentration of a stock solution of NO as measured by the quenching technique. The NO solution was first mixed with a deoxygenated buffer solution and then with solutions of Hb containing various concentrations of sodium hydrosulfite. All concentrations are μM

[Hb]	$[Na_2S_2O_4]$	[NO]
91.5	–	53.4
92.5	200	54.5
92.5	600	54.5
92.3	1200	55.0
92.2	1800	54.8

2.5.5. Reaction (11)

The fast reaction of NO with oxymyoglobin has a rate constant value $k = 37 \times 10^9$ M^{-1} s^{-1} [20]. As O_2 removal by hydrosulfite is a slow process, a similar reaction with oxyhemoglobin, can interfere significantly with the quantitative trapping of NO by Hb. A kinetic simulation (not shown) of NO binding to Hb in the presence of O_2, taking into account also Reaction (11) and using the rate constant reported for the oxymyoglobin reaction, indicated that: (1) a significant fraction of NO escapes detection as HbNO because of Reaction (11); and (2) NO recovery as HbNO approaches 100% if the rate of NO association to Hb is made faster by adequately increasing the protein concentration. The numerical prediction was checked by an experimental simulation of the effect of Reaction (11), as follows. Stock solutions of NO and O_2 were mixed in M1, and after 10 ms the reactants were mixed in M2 with Hb solutions of increasing concentrations at a constant concentration of hydrosulfite (550 μM). Reference experiments were carried out by replacing the O_2 solution with deoxygenated buffer. Assuming negligible NO auto-oxidation during 10-ms aging in the reaction loop, a decrease in NO recovery with respect to the amount measured in the reference experiments measures the interference of Reaction (11). The ratio $[NO]_{obs}/[NO]_0$, between the observed NO concentration and that of the stock solution, as determined in the reference experiments, approaches unity when the hemoglobin concentration is sufficiently high (Fig. 5).

2.5.6. Other reactions

Oxygen, and also other oxidants such as the products of Reaction (9), could react with HbNO yielding methemoglobin (Hb^+) during the time required to record the spectra. The spectra of Hb solutions and HbNO/Hb mixtures reaching the flow cell after the reduction of free and bound O_2 by 550 μM hydrosulfite were identical within error to the reference spectra of Hb, HbNO and their linear combinations (Fig. 2). To check if Reaction (12) was significant under the conditions of the quenching experiments, the following con-

trols were carried out. A stock solution of NO was mixed in M1 with a solution of Hb containing 550 μM hydrosulfite and after an aging time of 100 ms, the solution was mixed in M2 with solutions of O_2 of varying titers. The first mixing allowed quantitative trapping of NO by Hb. The second mixing exposed HbNO to O_2 and to the products of O_2 reduction by hydrosulfite. The spectrum of the mixture of Hb and HbNO was recorded at 30-s intervals after stopping the flow (Fig. 6). In particular, the absorbance at 630 nm did not indicate a significant production of methemoglobin. The NO concentrations determined under these conditions are compared in Fig. 6 with the concentration determined in the absence of added O_2.

It should also be mentioned that NO reacts with the thiol groups of hemoglobin in the presence of O_2 [21]. However, the rate of this reaction is too slow to interfere with the NO determination under the conditions of our experiments.

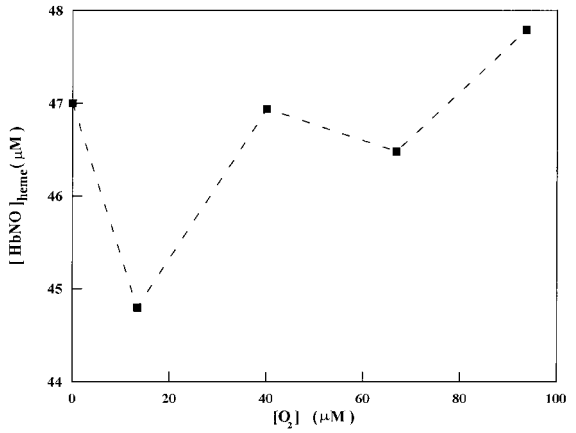

Fig. 6. (■), Concentration of nitrosylated hemes $[HbNO]_{heme}$, measured by mixing a stock solution of NO $[NO]_0 = 47$ μM, with a 150-μM solution of Hb, containing 550 μM hydrosulfite, in M1 (Fig. 1) and, after 100 ms, with solutions of varying concentrations of O_2 in M2. The time for complete O_2 reduction by hydrosulfite was 580 ms. The value $[NO]_0$, of the concentration of the stock solution was determined by replacing the oxygen solution with buffer deoxygenated by nitrogen tonometry.

3. Results

Fig. 7 is a typical progress curve for the auto-oxidation reaction of NO at 20°C in 50 mM phosphate buffer, pH 7. In such experiments the hydrosulfite concentration was kept constant at 550 μM and the ratio of hemoglobin to the sum of the NO plus O_2 concentrations was optimized as shown in Fig. 5.

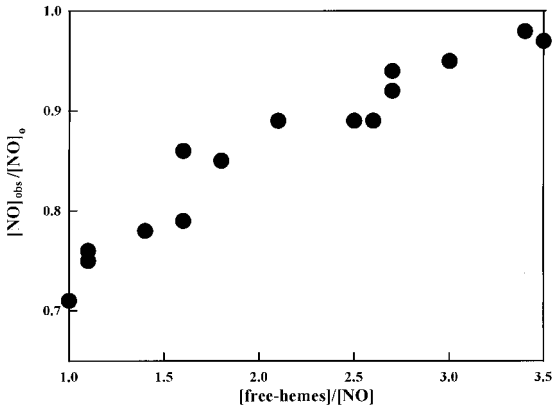

Fig. 5. Ratio $[NO]_{obs}/[NO]_0$ vs. the ratio [free hemes]/$[NO]_0$. $[NO]_{obs}$ was the NO concentration measured by mixing a solution of initial concentration $[NO]_0$ with a solution of O_2 of known titer in M1 and, after negligible auto-oxidation of NO in the reaction loop (10 ms), with Hb/hydrosulfite mixtures of varying Hb concentration, in M2. The concentration [free hemes] refers to the hemoglobin sites remaining vacant after binding all NO of the stock solution $[NO]_0$, and removing all O_2 by hydrosulfite.

Fig. 8 is a logarithmic plot of the initial velocities as obtained from the progress curves at a constant initial value of NO concentration (60 μM) and varying values of O_2 concentration, in the range 8–60 μM. Fig. 9 is a similar plot obtained at a constant initial value of O_2 concentration (60 μM) and varying values of NO concentration, in the range 12–100 μM. Each data point was the average value from five experiments. The slope value in Fig. 8, 0.98 ± 0.01, indicates a first order rate law with respect to oxygen. The slope value in Fig. 9, 2.099 ± 0.001, indicates a second order rate law with respect to NO. Thus the rate equation [22]:

$$v = -\mathrm{d}[NO]/\mathrm{d}t = -4k_{aq}[NO]^2[O_2]$$

$$= k[NO]^2[O_2] \quad (14)$$

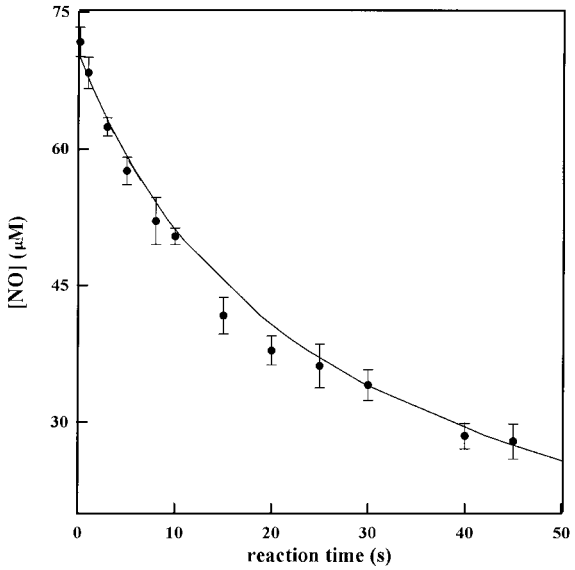

Fig. 7. Time course of decrease in NO concentration in the auto-oxidation reaction at 20°C in 50 mM phosphate, pH 7. The initial values of the reactant concentrations were: $[NO]_0 = 70.4 \pm 0.82$ μM and $[O_2]_0 = 60.0 \pm 2.0$ μM. The bars indicate the error as calculated by averaging the data from five measurements. The line was fitted assuming a third order rate constant, $k_{aq} = 2.23(\pm 0.26) \times 10^6$ M^{-2} s^{-1}, for the auto-oxidation reaction. Data at longer reaction times, up to 5 min, were fitted similarly using the same value of k_{aq}.

was fitted to progress curves to obtain a weighted average value for the third order rate constant $k_{aq} = 2.23(\pm 0.26) \times 10^6$ M^{-2} s^{-1} at pH 7 and 20°C. Such a value, obtained from kinetic data in the time range 10 ms–50 s, did not change significantly when the data were collected over a longer time range, up to several minutes. Measurements carried out at 20°C in 50-mm borate buffer, pH 9, yielded a value $k_{aq} = 2.57(\pm 0.11) \times 10^6$ M^{-2} s^{-1}.

4. Discussion

4.1. Validation of the method

Side reactions can occur in the quenching of the NO/O_2 mixture by the Hb/hydrosulfite mixture. Specific controls or theoretical and experimental simulations were carried out to check their effects on the correct estimation of NO not consumed in the auto-oxidation reaction.

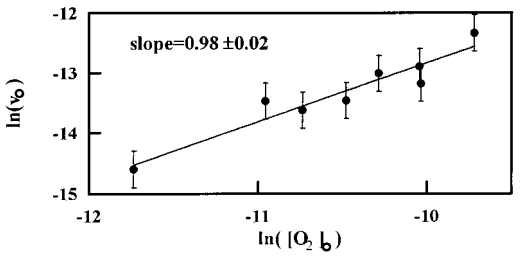

Fig. 8. Logarithmic plot of the initial velocity of the auto-oxidation reaction vs. the initial O_2 concentration, at 20°C, in 50 mM phosphate, pH 7. The bars indicate the error as calculated by averaging the data from five measurements.

The NO concentration of a stock solution in 50 mM phosphate, reported in Table 1, was reproducible within 2% of the value and independent of the concentration of hydrosulfite added to the quenching solution of Hb up to 1800 μM. The NO concentration value was within 10% of the value calculated using the NO solubility coefficient in water [23]. These tests indicate that, in the absence of the auto-oxidation reaction, NO dissolved in buffer was correctly estimated as HbNO in HbNO/Hb mixtures. As the O_2 reduction by hydrosulfite is a slow process, which leads to aggressive products, superoxide anion and hydrogen peroxide, the experiments in Fig. 6 were designed to check the effects of O_2, O_2^- and H_2O_2 on the absorption spectra of the Hb/HbNO mixture and on the determination of the HbNO concentration of a stock solution. The spectra were fitted by a linear combination of the spectra of Hb and HbNO with the same precision as in the absence of added O_2 (Fig. 2), suggesting that the peroxides did not influence the spectra of

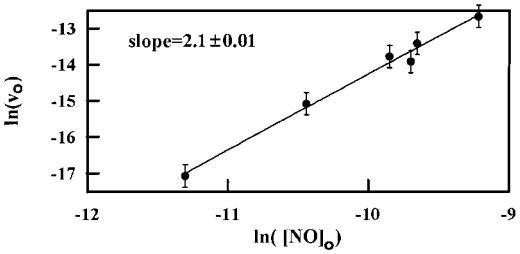

Fig. 9. Logarithmic plot of the initial velocity of the auto-oxidation reaction vs. the initial NO concentration, at 20°C, in 50 mM phosphate, pH 7. Bars as in Fig. 8.

the hemoglobin components significantly. A third component, such as Hb^+, produced by the reaction of O_2 and the peroxides with HbNO, may not be detected in the spectra observed 30 s after quenching due to Hb^+ reduction to Hb by hydrosulfite. However, the oxidation of HbNO, yielding Hb^+ and nitrite through Reaction (12), would bring about an apparent decrease in the NO concentration determined as HbNO. The data in Fig. 6 indicate that the determination of the NO concentration of the stock solution was the same in the presence as in the absence of O_2.

The crucial aspect of the procedure was that a significant fraction of NO not consumed in the auto-oxidation reaction could escape detection via Reaction (11), which oxidizes NO to nitrate. Kinetic simulations indicated that the problem could be circumvented by increasing the concentration of Hb to make NO binding to Hb, Reaction (7), faster than Reactions (9) and (11). The indications of the theoretical simulations were confirmed by experimental simulations, such as that shown in Fig. 5. The effect of the Hb concentration on Reactions (7), (8) and (11) was measured experimentally to obtain the Hb concentration value adequate for a full recovery of NO as HbNO. These experimental simulations, carried out over the range of O_2 concentrations used for the study of the kinetics of the auto-oxidation reaction, were a solid validation of the procedure.

4.2. Mechanism of NO auto-oxidation in aqueous medium

Methods for the direct electrochemical measurement of NO concentrations have been described and commercial NO sensors are available [14]. However, the technology is not yet satisfactory and the finding that the kinetics of NO auto-oxidation is zero order with respect to NO concentration through the use of a porphyrinic NO sensor is likely due to methodological problems related to the performance of the sensor. The method described in this work was based on the monitoring of the concentration of NO by a chemical quenching procedure using Hb as a quencher. Most of the known reactions that could interfere with such a quenching were considered in designing the kinetic experiments. The results support the mechanism indicated by other studies based on the measurement of the rate of formation of the final products of NO auto-oxidation in aqueous medium, i.e. H^+ and NO_2^-. Our study confirms that the reaction is first and second order with respect to O_2 and NO and that Reactions (1) and (2) are rate limiting with respect to Reaction (5). The value of the third order rate constant for the overall reaction, $k = 4 \times k_{aq} = 4 \times 2.23(\pm 0.26) \times 10^6\ M^{-2}\ s^{-1}$ is in line with values obtained by those studies: $k = 6(\pm 1.5) \times 10^6\ M^{-2}\ s^{-1}$, in 0.1 M phosphate, pH 7.4 and 22°C [9]; $k = 6.3(\pm 0.4) \times 10^6\ M^{-2}\ s^{-1}$, in 2 mM phosphate, pH 7.7 and 20°C [10]; $k = 8.8(\pm 0.4) \times 10^6\ M^{-2}\ s^{-1}$, at 15–30°C [12]; and $k = 4 \times k_{aq} = 4 \times 2.1(\pm 0.5) \times 10^6\ M^{-2}\ s^{-1}$, in 60 mM HCl, pH 1 and 25°C [13].

In conclusion, the method described in this work measures the rate of NO decrease accurately even in a difficult case such as the auto-oxidation reaction. It can find useful applications, possibly in combination with other methods, in the study of the mechanisms of various reactions of NO, e.g. the reactions involving peptides and proteins, for which the measurement of the rate of product formation is not straightforward.

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