Pulse-radiolysis Study of the Reaction of Nitric Oxide with Superoxide

Kazuo Kobayashi, Miyako Miki and Seiichi Tagawa
The Institute of Scientific and Industrial Research, Osaka University, Mihogaoka 8–1, Ibaraki, Osaka 567, Japan

Using the pulse-radiolysis technique, nitric oxide (NO) was produced by the reaction of an hydrated electron with NO_2^-. This was verified by the formation of the NO complex of iron(II) myoglobin (Mb) after pulse radiolysis of iron(III) myoglobin in the presence of NO_2^- under anaerobic conditions. By employing this technique, the reaction of superoxide (O_2^-) with NO was followed directly in an oxygen-saturated solution containing NO_2^-. It was found that O_2^- reacted with NO with a second-order rate constant of 3.8×10^9 dm^3 mol^{-1} s^{-1} at pH 7.5 to form oxoperoxonitrate(III) [$NO(O_2)^-$] which has an absorption maximum around 300 nm. Oxoperoxonitrate(III) thus formed decomposed with a first-order rate constant of 0.8 s^{-1} at pH 7.5. A similar decay process was observed in the reaction of O_2^- with NO by monitored stopped-flow, where one part KO_2 in dimethyl sulfoxide was mixed with nine parts of a NO-saturated solution in water. The formation of oxoperoxonitrate(III) was complete within the dead-time of the stopped-flow system.

Recently, nitric oxide (NO) has been shown to be involved in a variety of important biological functions, including vascular smooth muscle relaxation, neurotransmission and immune activation.[1–3] Despite the many recent discoveries involving NO, some of its basic chemistry is not well understood owing to a lack of information concerning its reactivity and reaction products. The spectral and kinetic behaviour of NO and its redox states are conveniently studied by pulse radiolysis.[4,5] With this technique, the reaction of NO with superoxide (hyperoxide) [equation 1], which appears to occur *in vivo*,[6,7]

$$O_2^- + NO \longrightarrow NO(O_2)^- \quad (1)$$

was observed directly.[4] However, it is difficult to determine the rate constant of the reaction upon pulse radiolysis in the presence of NO, since NO^- reacts to form various species such as $N_2O_2^-$. In fact, the rate constant (3.7×10^7 dm^3 mol^{-1} s^{-1}) determined by pulse radiolysis[4] is two orders of magnitude smaller than that obtained from flash photolysis (6.7×10^9 dm^3 mol^{-1} s^{-1}).[8] On the other hand, earlier radiation studies showed that an hydrated electron (e_{aq}^-) reacts with NO_2^- to form NO,[9–11] although there is no direct evidence for the formation of NO. It is possible to produce NO by pulse radiolysis of an aqueous solution of NO_2^- and to follow its reactions spectrophotometrically.

To confirm the formation of NO after pulse radiolysis of an aqueous solution of NO_2^-, we have studied the changes in absorbance with time in the presence of the haemoprotein myoglobin (Mb). It is well known that NO binds to iron(II) myoglobin to give a spectrum characteristic of NO–iron(II) myoglobin. In addition, by the use of this technique, the reaction was followed spectrophotometrically; the results are compared with those obtained from stopped-flow experiments where KO_2 in dimethyl sulfoxide is mixed with a NO-saturated buffer solution.

Experimental

Materials.—Commercially available horse myoglobin was used without further purification. Superoxide dismutase (SOD) was purified from the crude material (Sigma) by column chromatography (diethylaminoethylcellulose) according to the method of McCord and Fridovich.[12] All other reagents were of the highest purity available commercially.

Pulse Radiolysis.—Pulse-radiolysis experiments were performed with an electron linear accelerator at the Institute of Scientific and Industrial Research of Osaka University.[13–15] The pulse width and energy were 8 ns and 27 MeV ($\approx 4.23 \times 10^{-12}$ J), respectively. The dose was in the range of 15–100 Gy.

A sample of metmyoglobin for pulse radiolysis was prepared as follows. A solution containing 8×10^{-6} mol dm^{-3} metmyoglobin, 5×10^{-3} mol dm^{-3} phosphate buffer (pH 7.5), 1×10^{-3} mol dm^{-3} $NaNO_2$ and 0.1 mol dm^{-3} *tert*-butyl alcohol (scavenging $^{\bullet}OH$) was deoxygenated by repeated evacuation and flushing with argon. To study the reaction with O_2^-, a sample of (1–5) $\times 10^{-3}$ mol dm^{-3} $NaNO_2$ in 5×10^{-3} mol dm^{-3} phosphate buffer (pH 5–8), or 1×10^{-4} mol dm^{-3} borate buffer (pH 8–10) and 0.1 mol dm^{-3} sodium formate, or 0.1 mol dm^{-3} *tert*-butyl alcohol (scavenging $^{\bullet}OH$) was saturated with oxygen by bubbling with oxygen gas. The initial concentration of O_2^- generated was determined using $\varepsilon_{260} = 1925$ dm^3 mol^{-1} cm^{-1}.[16]

Stopped Flow.—The stopped-flow apparatus used was a Union Giken model RA-401 spectrometer capable of mixing nine parts of an aqueous solution with one part of Me_2SO.[17] A solution of NO-saturated buffer was prepared as follows. A sample cuvette containing 5×10^{-2} mol dm^{-3} phosphate buffer (pH 5–8) or 5×10^{-2} mol dm^{-3} borate buffer (pH > 8) was sealed with a rubber cap and deaerated by repeated cycles of evacuation and flushing with argon gas. Nitric oxide gas was injected anaerobically into the sample cuvette after evacuation. The NO-saturated buffer (2×10^{-3} mol dm^{-3}) was introduced into the large reservoir of the flow system anaerobically. A solution containing approximately (1–4) $\times 10^{-4}$ mol dm^{-3} of KO_2 and (2–8) $\times 10^{-4}$ mol dm^{-3} icosahydrodibenzo[*b*,*k*]-1,4,7,10,13,16-hexaoxacyclooctadecine (dicyclohexano-18-crown-6) in Me_2SO, as described by Valentine and Curtis,[18] was deoxygenated by evacuation and flushing with argon gas, and introduced into the small reservoir anaerobically. The NO-

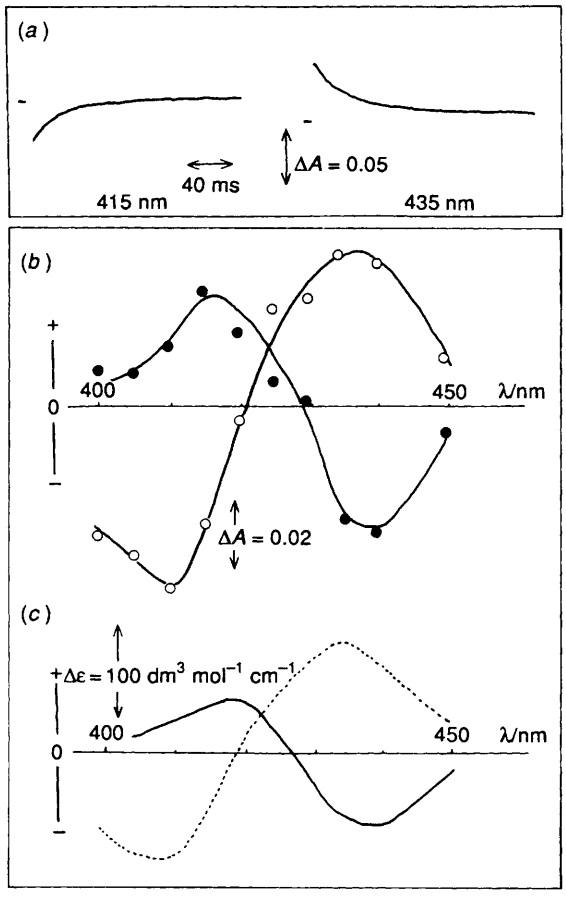

Fig. 1 (*a*) Absorption changes after pulse radiolysis of metmyoglobin in the presence of $NaNO_2$ at pH 7.5 measured at 415 and 435 nm; (*b*) kinetic difference spectra at 10 μs (○) and 100 ms (●) after pulse radiolysis of metmyoglobin; (*c*) difference spectra of met- minus deoxymyoglobin (····) and deoxymyoglobin minus NO–iron(II) myoglobin (——), respectively [5×10^{-3} mol dm^{-3} phosphate buffer (pH 7.5), 1×10^{-3} mol dm^{-3} $NaNO_2$, 0.1 mol dm^{-3} *tert*-butyl alcohol and 8×10^{-6} mol dm^{-3} metmyoglobin]

saturated buffer was rapidly mixed with the solution of KO_2 in the stopped-flow apparatus. The temperature was thermostatically controlled at 25 °C.

Spectroscopic Measurements.—Absorption spectra were measured using a Shimadzu MPS-2000 spectrometer. An iron(II)–NO complex of Mb was prepared by the use of nitrite and sodium dithionite according to Yonetani *et al.*[19]

Results

Formation of Nitric Oxide by Pulse Radiolysis.—To confirm the formation of NO from the reaction of e_{aq}^- with NO_2^- changes in absorbance with time were followed after pulse radiolysis of a solution of NO_2^- (1×10^{-3} mol dm^{-3}) in the presence of metmyoglobin (8×10^{-6} mol dm^{-3}) under anaerobic conditions. As shown in Fig. 1(*a*), the absorption spectrum at 415 nm shows a fast initial decrease followed by a slow increase, and at 435 nm the absorption increases and then decreases slowly. The difference spectrum at 10 μs after the pulse is shown in Fig. 1(*b*). It is similar (absorption maximum at 435 nm and minimum at 410 nm) to the difference spectrum of met- minus deoxy-myoglobin shown in Fig. 1(*c*). This suggests that the faster absorption change can be attributed to the reduction of the haem iron of Mb by e_{aq}^- [equation (2)]. In

$$Fe^{3+} + e_{aq}^- \longrightarrow Fe^{2+} \quad (2)$$

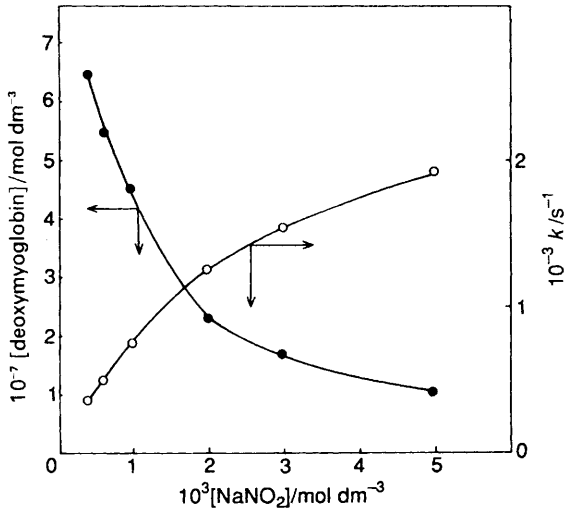

Fig. 2 Sodium nitrite concentration dependence of the formation of deoxymyoglobin (●) and the apparent rate constant of the formation of NO–iron(II) myoglobin Mb (○) [5×10^{-3} mol dm^{-3} phosphate buffer (pH 7.5), 0.1 mol dm^{-3} *tert*-butyl alcohol and 8×10^{-6} mol dm^{-3} metmyoglobin]

contrast, the difference spectrum of the slower phase, between 10 μs and 100 ms after the pulse, is similar to that of deoxymyoglobin minus the NO complex of iron(II) myoglobin. This suggests that the slower phase can be attributed to the binding of NO to the iron(II) haem of Mb [equation (3)]. Under

$$Fe^{2+} + NO \longrightarrow Fe^{2+}\text{–}NO \quad (3)$$

these conditions e_{aq}^- reacts with both metmyoglobin and NO_2^-. Therefore, it is confirmed that NO is generated by the reaction of e_{aq}^- with NO_2^-, as shown in equation (4). Initially

$$NO_2^- + e_{aq}^- \longrightarrow NO_2^{2-} \xrightarrow{H^+} NO + OH^- \quad (4)$$

5×10^{-7} mol dm^{-3} of metmyoglobin was reduced and the deoxymyoglobin thus formed combined with NO quantitatively. The concentrations of deoxymyoglobin and NO–iron(II) myoglobin generated after the pulse were estimated from the absorption changes shown in Fig. 1(*a*). The binding of NO to deoxymyoglobin obeyed first-order kinetics, suggesting that the concentration of NO generated is much higher than that of deoxymyoglobin under these conditions. However, the concentration of NO cannot be measured directly. Thus, the NO_2^- concentration dependence of the reactions occurring after the pulse was examined. The reduction yield of metmyoglobin decreased with an increase in the concentration of NO_2^- in the range 2×10^{-4}–5×10^{-3} mol dm^{-3} (Fig. 2). On the other hand, the apparent rate constant of the formation of NO–iron(II) myoglobin increases with the increase in the initial concentration of NO_2^- (Fig. 2). These results suggest that the reaction of e_{aq}^- with NO_2^- and the resulting production of NO increases with the increase in NO_2^-. From the second-order rate constant (1.7×10^7 dm^3 mol^{-1} s^{-1}) of reaction (3) reported previously,[20] the concentration of NO is estimated to be in the range (1.6–11.4) $\times 10^{-6}$ mol dm^{-3}. In the presence of higher concentrations of NO_2^- ($> 1 \times 10^{-2}$ mol dm^{-3}), metmyoglobin was not reduced but the binding of NO to metmyoglobin was observed (data not shown). However, the absorption change for this process was very small, due to the low affinity of NO for metmyoglobin.[20]

Reaction of NO *with* O_2^-.—In order to follow the reaction of NO with O_2^- we have studied the absorption change with time after pulse radiolysis of oxygen-saturated phosphate buffer in the presence of 5×10^{-3} mol dm^{-3} $NaNO_2$. As shown in Fig.

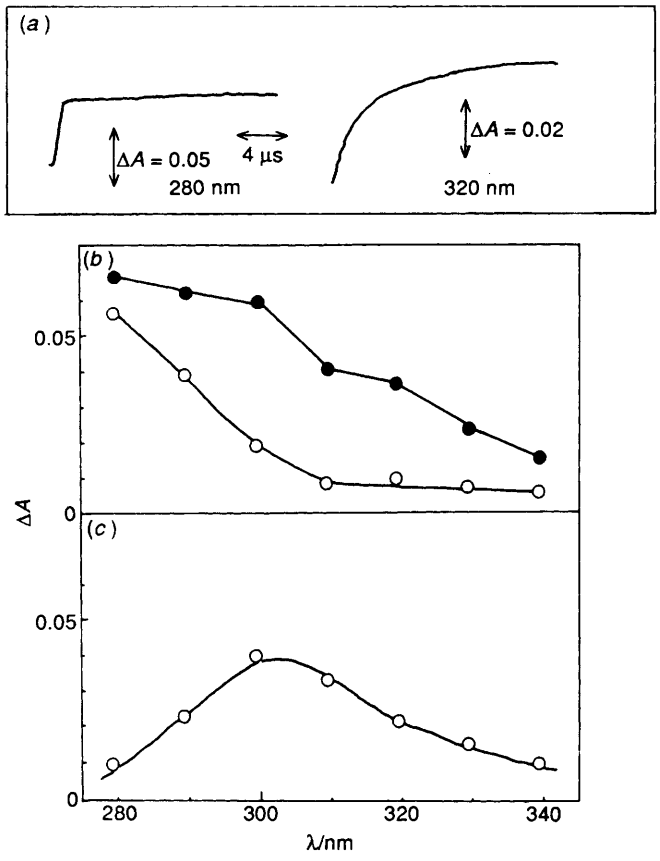

Fig. 3 (*a*) Absorption changes after pulse radiolysis of an aqueous solution of $NaNO_2$ with oxygen saturation at pH 7.5 measured at 280 and 320 nm. (*b*) Kinetic difference spectra at 400 ns (○) and 50 μs (●) after the pulse. (*c*) Difference spectrum between 400 ns and 50 μs after the pulse [5×10^{-3} mol dm^{-3} phosphate buffer (pH 7.5), 5×10^{-3} mol dm^3 $NaNO_2$ and 0.1 mol dm^{-3} sodium formate]

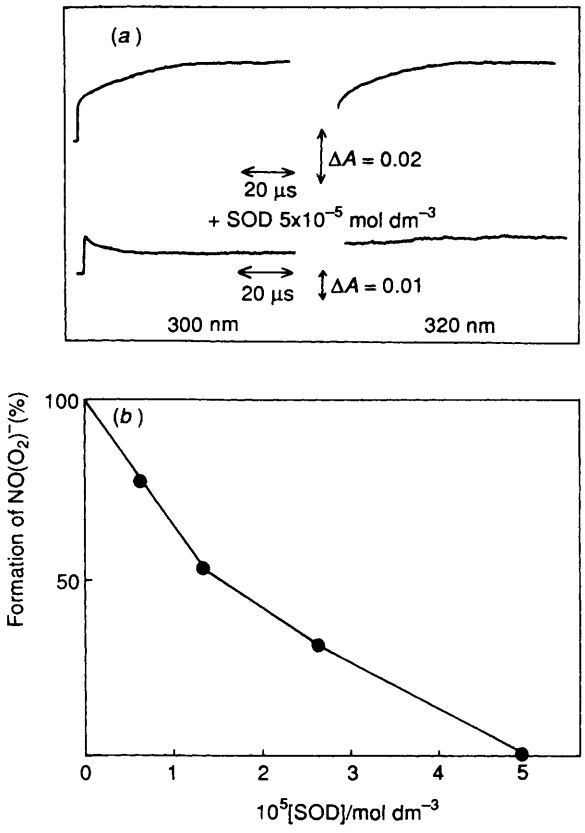

Fig. 4 (*a*) Absorption changes after pulse radiolysis of an aqueous solution of $NaNO_2$ with oxygen saturation in the absence and presence of superoxide dismutase. (*b*) Effect of superoxide dismutase on the formation of oxoperoxonitrate(III) [5×10^{-3} mol dm^{-3} phosphate buffer (pH 7.5), 5×10^{-3} mol dm^{-3} $NaNO_2$ and 0.1 mol dm^{-3} sodium formate]

3(*a*), the absorption at 280 nm increased rapidly and then more slowly, whereas only a slow absorption change was observed at 320 nm. The difference spectra at 400 ns and 50 μs after the pulse are shown in Fig. 3(*b*). The spectrum at 400 ns after the pulse is consistent with the formation of O_2^-, as shown in equation (5). The difference spectrum between 400 ns and 50 μs

$$e_{aq}^- + O_2 \longrightarrow O_2^- \qquad (5)$$

is shown in Fig. 3(*c*). The absorption maximum at around 300 nm is similar to that of oxoperoxonitrate(III) [$NO(O_2)^-$] in 0.1 mol dm^{-3} KOH.[21] Therefore, it is concluded that the slower absorption change is due to the reaction between O_2^- and NO [reaction (1)]. The absorption change in Fig. 3(*b*) reveals that 2.4×10^{-5} mol dm^{-3} $NO(O_2)^-$, calculated from its absorption coefficient ($\varepsilon_{302} = 1670$ dm^3 mol^{-1} cm^{-1}),[21] was formed in the solution initially containing 3.6×10^{-5} mol dm^{-3} O_2^-. Assuming that NO reacts with O_2^- quantitatively, the initial concentration of NO is estimated to be 2.4×10^{-5} mol dm^{-3}. The decay of the remaining O_2^- was observed in the time range of 10 ms (data not shown).

The formation of $NO(O_2)^-$ was found to be inhibited upon the addition of SOD, as shown in Fig. 4(*a*). In the presence of 5×10^{-5} mol dm^{-3} SOD, the rapid increase in the absorption at 300 nm was not affected whereas that at 320 nm showed no increase. Subsequently, the decay of O_2^- due to the enzymatic reaction of SOD was observed. Fig. 4(*b*) shows the dependence of the absorption change at 320 nm upon the concentration of SOD. Half-maximum inhibition was observed at 1.5×10^{-5} mol dm^{-3} SOD, when the concentrations of O_2^- and NO generated are approximately 5×10^{-5} and 1×10^{-5} mol dm^{-3}, respectively. This suggests that NO reacts with O_2^- at a rate comparable to that of SOD.

On pulse radiolysis (10–100 Gy) of an O_2-saturated solution containing 1×10^{-3} mol dm^{-3} $NaNO_2$ the reaction of O_2^- with NO obeyed pseudo-first-order kinetics. Assuming the stoichiometry of reaction (1), the concentration of NO can be estimated to be about 10–20 times lower than that of O_2^- at corresponding doses. Under these conditions an excess of O_2^- is generated, and therefore a competition between the disproportionation of O_2^- and the reaction of NO with O_2^- occurs. However, the disproportionation of O_2^- does not interfere with reaction (1) since its rate is much faster than that of the disproportionation. Fig. 5 shows the dependence of the pseudo-first-order rate constant on the concentration of O_2^-. From the slope, the second-order rate constant in reaction (1) is calculated to be 3.8×10^9 dm^3 mol^{-1} s^{-1} at pH 7.5.

Decay of Oxoperoxonitrate(III).—Oxoperoxonitrate(III) was found to decay in the time range of 1 s. Typical examples are shown in Fig. 6(*a*). The decay obeyed first-order kinetics (0.8 s^{-1} at pH 7.5) and was independent of the concentration of $NO(O_2)^-$. Above pH 11, $NO(O_2)^-$ was very stable and could be kept for 5 min without changes in the absorption spectrum. To test the contribution of transition metals to the decay of $NO(O_2)^-$, the effect of edta (ethylenediaminetetraacetate) and iron(III)–edta were examined; neither (100 μmol dm^{-3}) had any effect.

The reaction of NO with O_2^- was also examined by the stopped-flow kinetic method. When the NO-saturated buffer was mixed with O_2^- a rapid increase in absorbance at 320 nm occurred followed by a first-order decay process similar to that in Fig. 6(*a*). A spectrum similar to that of Fig. 3(*c*) was obtained

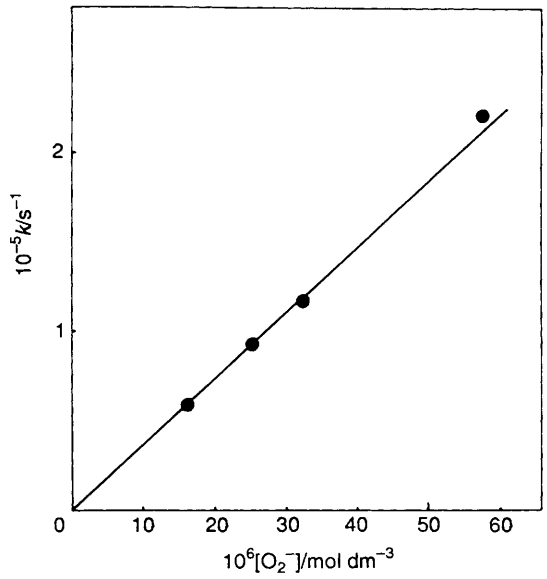

Fig. 5 Dependence of O_2^- concentration on the pseudo-first-order rate constant for the reaction of NO with O_2^- [5 × 10^{-3} mol dm^{-3} phosphate buffer (pH 7.5), 0.1 mol dm^{-3} sodium formate and 1 × 10^{-3} mol dm^{-3} $NaNO_2$]

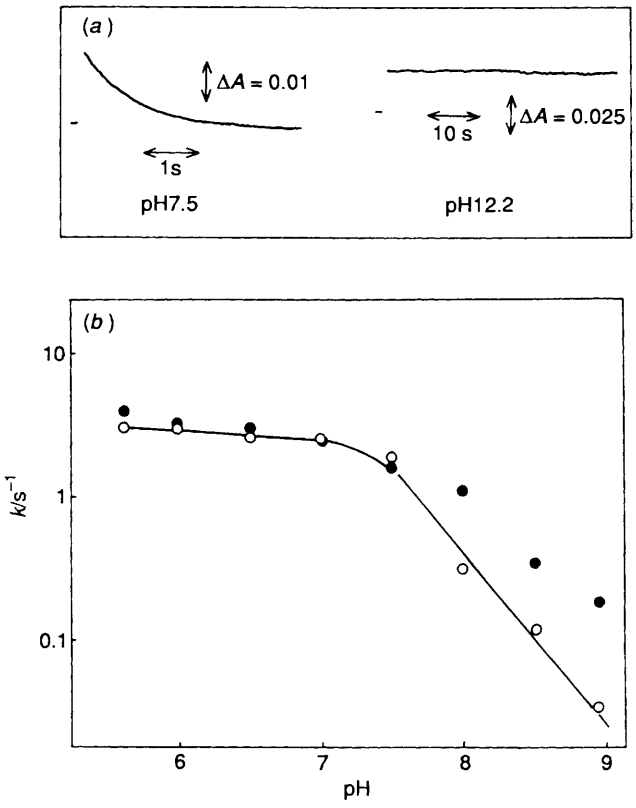

Fig. 6 (*a*) Absorption changes after pulse radiolysis of an aqueous solution of $NaNO_2$ with oxygen saturation at pH 7.5 and 12.2 measured at 320 nm. (*b*) pH Dependence of the rate constants for decay of $NO(O_2)^-$ determined by pulse radiolysis (●) [5 × 10^{-3} mol dm^{-3} phosphate buffer (pH 5.5–8), or 1 × 10^{-2} mol dm^{-3} borate (above pH 8), 5 × 10^{-3} mol dm^{-3} $NaNO_2$ and 0.1 mol dm^{-3} *tert*-butyl alcohol with oxygen saturation] and stopped-flow methods (○) [1.2 × 10^{-4} mol dm^{-3} KO_2, 2.5 × 10^{-4} mol dm^{-3} dicyclohexano-18-crown-6, and the final reaction mixture was 10% (v/v) Me_2SO; 5 × 10^{-2} mol dm^{-3} phosphate buffer (pH 5.5–8) and 5 × 10^{-2} mol dm^{-3} borate (above pH 8)]

immediately after mixing (data not shown), indicating that $NO(O_2)^-$ was formed in this system. The development of the 320 nm absorbance was complete within the dead-time of the stopped-flow system (≈2–3 ms). Therefore, the rate constant of reaction (1) cannot be determined by the stopped-flow method. We can merely say that the rate should be larger than 10^7 dm^3 mol^{-1} s^{-1}.

The effect of pH on the decay of $NO(O_2)^-$ was examined by pulse radiolysis and stopped-flow methods. Fig. 6(*b*) shows the pH dependence of the rate constant for the decay of $NO(O_2)^-$. The rate constants obtained were similar for both methods. The values increased with decreasing pH from 8.5 to 7.0 and became constant below pH 7.0. Below pH 5.5 the rate constant could not be determined since the absorption of $NO(O_2)^-$ at 300 nm was very small (see below).

The absorption changes at 320 nm, after the pulse, were noticeably decreased with a decrease in pH, though the rate of the formation of $NO(O_2)^-$ was not affected. This result cannot be explained in terms of pH-dependent formation of NO by the pulse technique, since a similar result was obtained by the stopped-flow method. Fig. 7(*a*) and 7(*b*) show pH-dependent absorption changes at 320 nm obtained by the pulse radiolysis and stopped-flow methods, respectively. The pK value of this transition is found to be approximately 6.8. The effect of pH cannot be explained by the difference in reactivity of HO_2 and O_2^- toward NO, since the dissociation of HO_2 is much more acidic (pH 4.88).[22] The alternative possibility, that the effect of pH on the spectra is due to the concentration of $NO(O_2)^-$ formed, can be excluded, since the amount of reduced nicotinamide adenine dinucleotide (NADH) oxidised by $NO(O_2)^-$ was not affected below pH 7.[23] The effect of pH can be attributed to the difference in the spectra between $NO(O_2)^-$ and $NO(O_2H)$. The spectrum of $NO(O_2H)$, obtained from the reaction of OH with NO_2 by pulse radiolysis, has an absorption maximum at 240 nm with little absorption around 300 nm.[24]

Discussion

Current experiments show that NO is formed by the reaction of e_{aq}^- with NO_2^-, which is verified by the formation of NO–iron(II) myoglobin. However, this reaction can be interpreted by the sequence of events shown in reaction (4). An initial step is the formation of NO_2^{2-}, and then rapid association of H^+ and a loss of OH^- leads to the final product, NO. Grätzel *et al.*[10] proposed that NO_2^{2-} (absorption maximum at 260 nm) is formed as an intermediate, followed by decay of NO_2^{2-} with a half-life of 12 μs at low concentrations of phosphate buffer (10^{-4} mol dm^{-3}). In contrast, we did not observe any such decay process in 10^{-2} mol dm^{-3} phosphate buffer (data not shown). The difference can be explained by the assumption that the conversion of NO_2^{2-} into NO is catalysed by phosphate ion, as proposed by Grätzel *et al.*[10] Therefore, it can be said that NO_2^{2-} does not contribute to the reaction observed in the time range of μs after the pulse under our experimental conditions.

The pulse radiolysis used here has permitted a direct demonstration of the reaction of NO with O_2^-. The second-order rate constant in reaction (1) is two orders of magnitude greater than that reported before (3.7 × 10^7 dm^3 mol^{-1} s^{-1}).[4] The discrepancy between this result and the study of Saran *et al.*[4] is considered to arise from the experimental conditions. Saran *et al.* followed the change in absorption with time of an aerobic solution containing NO. Under these conditions NO^- is formed, due to the reaction of e_{aq}^- with NO, and its subsequent reactions to form various species such as $N_2O_2^-$ therefore make it difficult to determine the rate constant. In our experiments NO and O_2^- can be produced concomitantly after the pulse. In addition, the reaction of NO with O_2 prior to the pulse can be avoided. Recently,[8] a similar rate constant was obtained by flash photolysis of an air-saturated nitrite solution. However, the value (6.7 × 10^9 dm^3 mol^{-1} s^{-1}) is about twice as

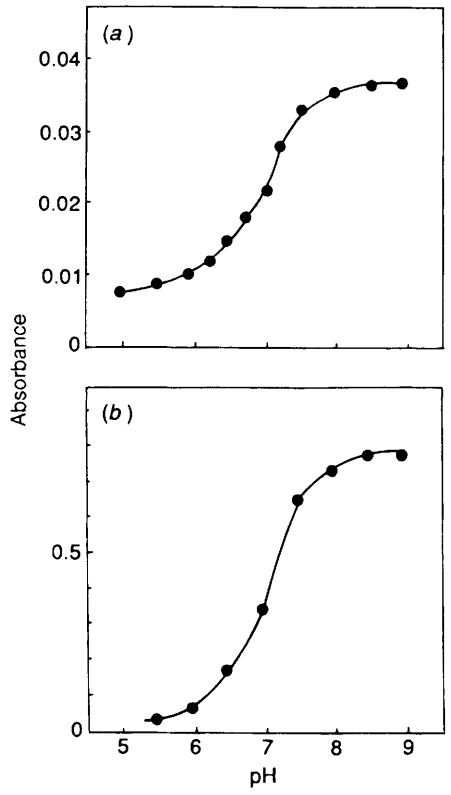

Fig. 7 pH Dependence of the absorption changes at 320 nm after pulse radiolysis of an aqueous solution of $NaNO_2$ with oxygen saturation (*a*) and after mixing of NO-saturated buffer with KO_2 (*b*). Experimental conditions are the same as for Fig. 6

high as that of our present method. The reason for the difference is as yet unknown. In these results,[8] the data were fitted by second-order kinetics, and this led to the estimated uncertainty in the rate constant. In contrast, by the use of pulse radiolysis, the concentrations of O_2^- and NO generated in the sample can be controlled, when the concentration of NO_2^- is changed. A linear plot of the pseudo-first-order constant *vs.* $[O_2^-]$ was obtained (Fig. 5) when the first-order condition ($[O_2^-] \gg [NO]$) was maintained. The uncertainty in the rate constants is estimated to be ± 10%.

The rate constant of reaction (1) is the highest value measured for the reaction of O_2^- with biological molecules, and is greater than that of Cu or Zn superoxide dismutase (2×10^9 dm^3 mol^{-1} s^{-1} at pH 7.0).[25] In fact, reaction (1) was inhibited completely at concentrations up to 5×10^{-4} mol dm^{-3} of SOD. This suggests that a (5–10) $\times$ 10^{-6} mol dm^{-3} level of SOD in tissue[26] would not effectively prevent reaction (1), if sufficient NO is released from the cells. Therefore, it can be said that reaction (1) appears to be physiologically significant.

Many reports suggest that $NO(O_2)^-$ is a strongly oxidizing species.[6,7] It is known to initiate oxidation of protein sulfhydryl,[27] lipid peroxidation[28] and DNA cleavage.[29] In most studies, however, $NO(O_2)^-$ is prepared by the reaction of nitrite and hydrogen peroxide with acid followed by quenching with base.[30] Under these conditions, there is the possibility of contamination by other reactive species. Reported syntheses of $NO(O_2)^-$ include a variety of techniques, *cf.* hydrogen peroxide and nitrite ester in base[31] and solid-state preparation by UV photolysis of potassium nitrite.[29] In contrast, NO and O_2^- can be produced concomitantly by the present method. Therefore, pulse radiolysis is a powerful tool for investigating the reactivity of $NO(O_2)^-$ toward various molecules.

Acknowledgements

This study was supported by a grant from the Naito Foundation (to K. K.). We thank the members of the Radiation Laboratory at the Institute of Scientific and Industrial Research, Osaka University, for their assistance in operating the accelerator.

References

1 L. J. Ignarro, *Annu. Rev. Pharmacol. Toxicol.*, 1990, **30**, 535.
2 S. Moncada, R. M. J. Palmer and E. A. Higg, *Pharmacol. Rev.*, 1991, **43**, 109.
3 E. Culotta and D. E. Koshland, jun., *Science*, 1992, **258**, 1862.
4 M. Saran, C. Michel and W. Bors, *Free Radical Res. Commun.*, 1990, **10**, 221.
5 G. Czapski, J. Holcman and B. H. J. Bielski, *J. Am. Chem. Soc.*, 1994, **116**, 11465.
6 J. S. Beckmann, T. W. Beckman, J. Chen, P. A. Masshall and B. A. Freeman, *Proc. Natl. Acad. Sci. U. S. A.*, 1990, **87**, 1620.
7 H. Ischiropoulos, L. Zhu, J. Chen, H. M. Tsai, J. C. Martin, C. D. Smith and J. S. Beckman, *Arch. Biochem. Biophys.*, 1992, **298**, 446.
8 R. E. Huie and S. Padmaja, *Free Radical Res. Commun.*, 1993, **18**, 195.
9 W. A. Seddon and H. A. Sutton, *Trans. Faraday Soc.*, 1963, **59**, 2323.
10 M. Grätzel, H. Henglein, J. Lilie and G. Beck, *Ber. Bunsenges. Phys. Chem.*, 1969, **73**, 646.
11 A. J. Elliot and A. S. Simons, *Can. J. Chem.*, 1984, **62**, 1831.
12 J. M. McCord and I. Fridovich, *J. Biol. Chem.*, 1969, **244**, 6049.
13 K. Kobayashi, M. Miki, K. Okamoto and T. Nishino, *J. Biol. Chem.*, 1993, **268**, 24642.
14 K. Kobayashi, Y. Harada and K. Hayashi, *Biochemistry*, 1991, **30**, 8310.
15 K. Kobayashi, K. Hayashi and M. Sono, *J. Biol. Chem.*, 1989, **264**, 15280.
16 B. H. J. Bielski, *Photochem. Photobiol.*, 1978, **28**, 645.
17 K. Kobayashi, T. Iwamoto and K. Honda, *Biochem. Biophys. Res. Commun.*, 1994, **201**, 1348.
18 J. S. Valentine and A. B. Curtis, *J. Am. Chem. Soc.*, 1975, **97**, 224.
19 T. Yonetani, H. Yamamoto, J. E. Erman, J. S. Leigh and G. H. Reed, *J. Biol. Chem.*, 1972, **247**, 2447.
20 E. J. Rose and B. M. Hoffman, *J. Am. Chem. Soc.*, 1983, **105**, 2866.
21 N. V. Blough and O. C. Zafiriou, *Inorg. Chem.*, 1984, **24**, 3504.
22 D. Behar, G. Czapski, J. Rabani, L. M. Dorfman and H. A. Schwarz, *J. Phys. Chem.*, 1970, **74**, 3209.
23 K. Kobayashi, unpublished work.
24 T. Løgager and K. Sehested, *J. Phys. Chem.*, 1993, **97**, 6664.
25 D. Klug, J. Rabani and I. Fridovich, *J. Biol. Chem.*, 1972, **247**, 4839.
26 C. C. Winterbourn, R. E. Hawkins, M. Brian and R. W. Carrel, *J. Lab. Clin. Med.*, 1975, **85**, 337.
27 R. Radi, J. S. Beckmanm, K. M. Bush and B. A. Freeman, *J. Biol. Chem.*, 1991, **266**, 4224.
28 N. Hogg, U. M. Darley-Usmar, M. T. Wilson and S. Moncada, *Biochem. Soc. Trans.*, 1993, **21**, 358.
29 P. A. King, V. E. Anderson, J. O. Edwards, G. Gustafso, R. C. Plumb and J. W. Suggs, *J. Am. Chem. Soc.*, 1992, **114**, 5340.
30 M. N. Hughes and H. G. Nicklin, *J. Chem. Soc. A*, 1968, 450.
31 J. R. Leis, M. E. Peña and A. J. Ríos, *J. Chem. Soc., Chem. Commun.*, 1993, 1298.

Received 13*th December* 1994; *Paper* 4/07580C