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REACTION OF NO WITH O_2^-. IMPLICATIONS FOR THE ACTION OF ENDOTHELIUM-DERIVED RELAXING FACTOR (EDRF)*

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Under physiological pH conditions (pH 7.2-7.4) the rate constant of the reaction NO + O_2^- yielding peroxonitrite ($ONOO^-$) was determined as $k = (3.7 \pm 1.1) \times 10^7\ M^{-1} s^{-1}$. The decay of peroxonitrite at this pH follows first order kinetics with a rate constant of 1.4 s^{-1}. At alkaline pH peroxonitrite is practically stable.

Possible consequences of these reactions for the biological lifetime of EDRF will be discussed.

KEY WORDS: Superoxide anion, nitric oxide, peroxonitrite, endothelial derived relaxation factor, biological messenging.

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INTRODUCTION

It has been shown recently[1-4] that nitric oxide (NO), which is derived from the terminal guanidino group of arginine,[4,5] serves as a vascular relaxant (endothelial derived relaxing factor, EDRF). It interacts with the heme group of soluble guanylate cyclase,[6] thereby interfering with Ca^{2+} -dependent processes in the target cell. Furthermore, superoxide dismutase (SOD) was shown to enhance the biological lifetime of NO[7,8]. If it is assumed that in this context SOD exerts its normal function of scavenging O_2^-, three different processes could be envisaged to explain the observed effects:

1) O_2^- reacts directly with NO thereby suppressing the message 'relaxation'; then it should be true
a) that O_2^- and NO react effectively with each other and
b) that the reaction product is inert, at least does *not* exert a 'relaxing'function;

2) O_2^- reacts with the *same* target site as NO, i.e. the heme group of soluble guanylate cyclase, in a competitive way thereby inhibiting the relaxing message of NO;

3) O_2^- carries the message 'constriction' to a receptor site which is *different* from the site of action of NO.

A verification of one of these three possibilities would have fundamental consequences for our understanding of the biological function of O_2^-. It would imply that O_2^- is actively produced and used by a much more diverse group of cells than just those involved in phagocytosis and thus may have a more general role in cell physiology,

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e.g. serve as a biological messenger molecule. This hypothesis has recently been put forward.[9-11] The chemistry of peroxonitrite and its decay mechanisms have been investigated previously,[12-15] but observations on the direct reaction between O_2^- and NO are scarce[16] and no kinetic measurements have been performed thus far to determine the rate constant directly. We therefore decided

1) to re-investigate the NO/O_2^--reaction by pulse radiolysis and

2) to determine whether these species would react competitively with a 'model heme' FTMP (Iron(III) tetrakis-(4-N-methyl-pyridyl)porphine).

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MATERIALS AND METHODS

NO was purchased from Linde (99.8% purity). All other chemicals were of the highest commercially available purity and used as supplied. Solutions were prepared with water from a Millipore Milli-Q system.

To obtain NO saturated solutions a stream of NO, which had passed a cool trap in dry ice/acetone slurry and a wash flask containing 50% KOH, was forced through ceramic sinters into an aqueous solution, which was previously deoxygenated by 30 minutes bubbling with N_2. When 10^{-4} M DTPA (diethylenetriamine penta-acetic acid) was added, these solutions were stable, i.e. no NO_2^- or other products of autoxidation could be detected for at least the time of one experimental run of 2 hours.

Within seconds after the NO-solution was mixed in a syringe-driven automated mixing system with any of the solutions indicated in the legends of the pertinent figures, it was irradiated by a pulse from the accelerator and the time dependent spectral changes at 15 different wavelengths were recorded at times ranging from 10 μs to up to 20 s after the pulse (all other experimental details have been described previously[17]).

RESULTS AND DISCUSSION

From radiolysis experiments it is known that all primary radiolysis products of water react with NO at diffusion controlled rates[18-20] (see Scheme I, upper left part). Under slightly acidic conditions the sole stable products in irradiated anaerobic NO-solutions are N_2O and NO_2^- which are produced with radiation chemical yields of 3.2 and 6.1 respectively[21] (lower left part of scheme). Under physiological pH-conditions the addition product of the hydrated electron, NO^-, reacts in a sequence of consecutively slower reactions with further NO to produce $N_2O_2^-$ and $N_3O_3^-$ which may eventually yield N_2O and NO_2^- (central part of the scheme).

Under aerobic conditions O_2 competes with NO for the electron and depending on the concentration ratios of NO and O_2 either the NO^--path or the path leading to O_2^--formation will be favored. In the latter case peroxonitrite should be observable if its formation proceeded to an appreciable extent.

Some remarks referring to the encircled numbers in the reaction scheme require attention:

1) Homolytic cleavage of $ONOO^-$ into the radicals NO_2 and OH, as proposed earlier, was questioned by Hughes and Nicklin[12] because of insufficient activation

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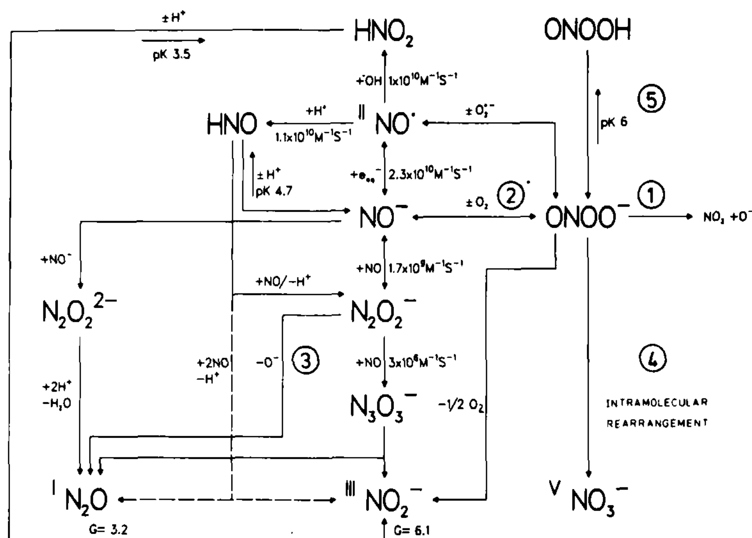

SCHEME I

energy. Under our experimental conditions no indications for this reaction have been found.

2) The reaction of NO with O_2, as suggested by Hughes and Nicklin[23,24], possibly occurs to a minor extent but since the absorptions of NO^- and O_2^- completely overlap around 260 nm, we were thus far not able to calculate its exact contribution to overall peroxonitrite formation.

3) The reaction $N_2O_2^- \rightarrow N_2O + O^-$, which would produce OH-radicals, has been suggested[20]. Taking a value of $3.5 \times 10^2\ s^{-1}$ for this reaction, as compared to 3×10^6 $M^{-1}s^{-1}$ for the competing reaction $N_2O_2^- + NO \rightarrow N_3O_3^-$, one would calculate that OH-production might become relevant at NO concentrations below 0.2 mM. Since the concentrations we had to use for our kinetic analysis were too low to analyze quantitatively for N_2O and NO_2^--formation we could not investigate this proposal in detail.

4) Some confusion exists in the literature on the values for the intramolecular rearrangement of $ONOO^-$ to NO_3^-. Values between $10^{-5}\ s^{-1}$ in strong alkali[14,16] up to $2 \times 10^3\ s^{-1}$ for the reaction of the acidic form HOONO[13] have been reported. The value of $1.4\ s^{-1}$ we now obtained in neutral solution agrees well with $1.5\ s^{-1}$ given by Barat *et al.*[25] or $1\ s^{-1}$ given by Wagner *et al.*[15]

5) The pK of HONOO was originally determined by Hughes and Nicklin[12] as 8.3 whereas later investigators found values of 5.3[13] and 6[25] which seem more reliable.

Since the absorption data of all intermediates in these reactions were known, the kinetics of peroxonitrite formation could be determined. Figure 1a gives the time dependence of the absorption decay around 260 nm in a system that only contains O_2^-. As can be seen O_2^- at this pH is rather stable; between 10 μs and 10 ms only a minor absorption decay occurs whereas after about 2 seconds all O_2^- has vanished.

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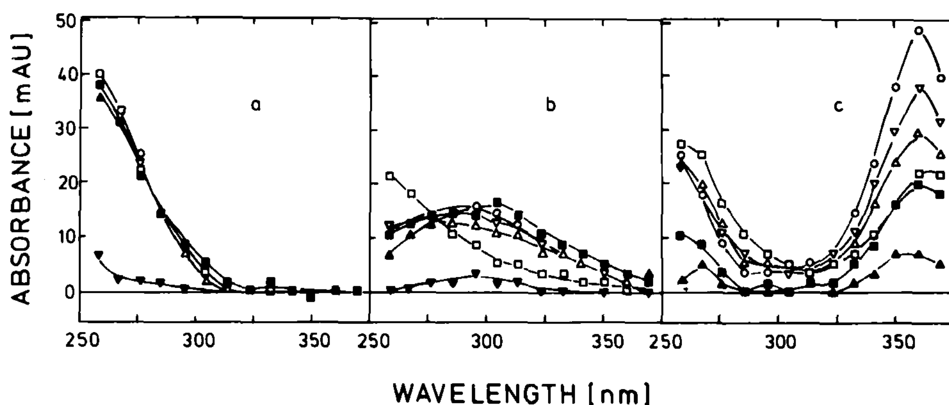

Fig. 1a	b	c
DECAY OF O_2^-	BUILDUP AND DECAY OF PEROXONITRITE	DECAY OF NO^- (LEFT) BUILDUP AND DECAY OF $N_2O_2^-$ (RIGHT)
Formate $1x10^{-2}M$ DTPA $1x10^{-4}M$ Phosphate buffer $3x10^{-2}M$ O_2 $1.23x10^{-3}M$ pH 7.4, Dose 37 Gy	Formate $1x10^{-2}M$ DTPA $1x10^{-4}M$ Phosphate buffer $3x10^{-2}M$ O_2 $7.3x10^{-4}M$ NO $8x10^{-4}M$ pH 7.4, Dose 37 Gy	Formate $1x10^{-2}M$ DTPA $1x10^{-4}M$ Phosphate buffer $3x10^{-2}M$ NO $2x10^{-3}M$ pH 7.4, Dose 37 Gy

TIME AFTER PULSE: □ 10/us △ 50/us ▽ 100/us ○ 500/us ■ 5.5ms ▲ 10.5ms ▼ 1.9 s

FIGURE 1

Figure 1c, for solutions only containing NO, shows that the absorption of NO at 260 nm, due to fast electron addition to NO, is already at its maximum after 10 μs whereas only a minor amount of N_2O_2, absorbing around 370 nm, has formed (open squares); then the NO -peak decreases while further N_2O_2 is formed. At about 500 μs (open circles) $N_2O_2^-$ is at its maximum and then decays until at 1.9 s (symbols not given in the Figure) both absorptions are at zero. Figure 1b accounts for the buildup and decay of peroxonitrite peaking around 302 nm. At the fastest time (10 μs) still some absorption due to O_2 and NO (which absorb at the same wavelength) can be seen (open squares), then the absorption of ONOO grows in to reach a maximum at 5.5 ms (filled squares) which then decays slowly to be almost zero after 1.9 s at this pH. At alkaline pH the decay becomes slower and at pH 11.4 no absorption decrease can be detected during the maximal observation time of 19 s (data not shown). Mathematical analysis of the data (for details see[17]) showed that the decay of NO^- and buildup of $N_2O_2^-$ in Figure 1c are kinetically correlated and from analysis of the peroxonitrite buildup at 302 nm (Figure 1b) the value of $(3.7 \pm 1.1) \times 10^7\ M^{-1}\ s^{-1}$ was derived for the reaction of O_2 with NO. The rate constant of the reaction of O_2 with FTMP was in a different set of experiments determined as $3 \times 10^9\ M^{-1}\ s^{-1}$. This value is higher than that obtained by others[22] under different ionic strength con-

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ditions. Based on our value we calculated from competition experiments a rate constant of $3.4 \times 10^7 M^{-1} s^{-1}$ for peroxonitrite formation which agrees reasonably well with the value obtained by direct spectral observation.

It is of interest to note, that the left part of the scheme does not predict nitrate formation. The only reaction pathway corroborated thus far to produce nitrate is via intermediary peroxonitrite formation (right part of scheme). The observation of NO_2^- *and* NO_3^- being produced from NO in activated phagocytic[26-28] and other [26,29] cells suggests peroxonitrite as an intermediate not only in phagocytosis but also in other cellular processes.

CONCLUSIONS

The reaction of O_2^- with NO yielding peroxonitrite proceeds with a rate of $(3.7 \pm 1.1) \times 10^7 M^{-1} s^{-1}$. At neutral pH peroxonitrite decays by a process which is governed by the slow first order rearrangement to NO_3^- ($k = 1.4 s^{-1}$). Under radiolytic conditions there is evidence for the reaction $NO^- + O_2$ to occur – which also leads to $ONOO^-$ – but thus far no exact rate constant could be determined and no biological consequences can be envisaged for this process.

The reaction of O_2^- with the heme model compound FTMP is very fast under our conditons ($k = 3 \times 10^9 M^{-1} s^{-1}$); no reaction of $ONOO^-$ with FTMP could be detected.

The present knowledge of the *chemistry* of the reaction $O_2^- + NO$ does not as yet allow conclusions concerning the influence of SOD on the 'biological' lifetime of EDRF and it cannot be decided from our chemical data which of the three possible pathways of NO/O_2^--messenging is effective. It is most interesting that O_2^- reacts with our model heme at a rate which is about one hundred times faster than that of its reaction with NO. This can only be meaningfully interpreted if the true *in vivo* concentrations of NO, O_2^- and guanylate cyclase are known and further elucidation must await investigations under truly physiological conditions.

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