OXIDATION OF HYDROXYLAMINE TO NITRITE AS AN ASSAY FOR THE COMBINED PRESENCE OF SUPEROXIDE ANIONS AND HYDROXYL RADICALS

W. Bors, E. Lengfelder[+], M. Saran
with the technical collaboration of
C. Michel, C. Fuchs[+] and C. Frenzel

Abteilung für Strahlenbiologie, Institut für Biologie
Gesellschaft für Strahlen- und Umweltforschung, 8042 Neuherberg

[+]Strahlenbiologisches Institut der Universität, 8 München 2,
Bavariaring 19, Germany (W.)

Received March 7,1977

Summary: The pulse-radiolytic oxidation of hydroxylamine by either hydroxyl radicals (OH), superoxide anions (O_2^-), or a combination of both radicals was investigated. It was found that only OH radicals efficiently attack the substrate, while O_2^- is necessary for the subsequent formation of nitrite. Determination of the latter reaction thus allows the detection of the combined presence of both oxygen radical species.

Introduction: The addition of hydroxylamine to illuminated chloroplasts resulted in the oxidation of this compound to nitrite (1), at the same time inhibiting photosynthetic electron transport (2). It was subsequently found, that the oxidation could be inhibited by superoxide dismutase (EC 1.15.1.1) (3,4). As a result, ELSTNER and HEUPEL (5) introduced the generation of nitrite from hydroxylamine, combined with the inhibition by superoxide dismutase, as a specific and sensitive assay for superoxide anions (O_2^-). The actual stoichiometry of the proposed reaction (4),

$$/1/ \qquad NH_2OH + 2\ O_2^- + H^+ \longrightarrow NO_2^- + H_2O_2 + H_2O$$

however, was not determined. We therefore considered it necessary to investigate the reaction of O_2^- with NH_2OH in detail, applying pulse radiolysis in combination with kinetic spectroscopy.

The surprising results were, that under pulse-radiolytic conditions almost

ISSN 0006-291X

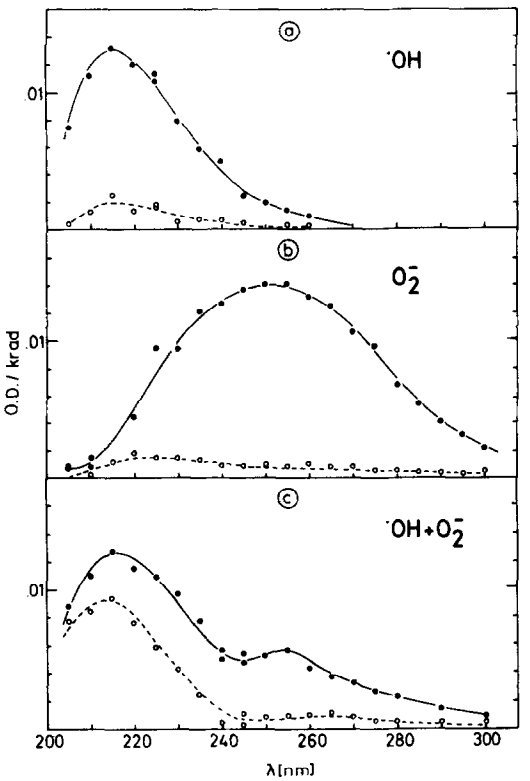

Figure 1 - Transient spectra of hydroxylamine (10^{-3} M, neutral solutions)
(a) N_2O-saturated solutions
(b) O_2^--saturated solutions containing HCOONa (10^{-2} M)
(c) O_2^--saturated solutions

● transient spectra after 20 usec
○ end spectra: (a) - 18 msec, (b) - 460 msec, (c) - 63 msec

no reaction of O_2^- with NH_2OH takes place in neutral solutions; NO_2^- was formed to an appreciable amount only in oxygenated solutions where both O_2^- and hydroxyl radicals (OH) were present. The consequence of these findings leads us to suggest the oxidation of hydroxylamine to nitrite as a specific test for the combined presence of OH and O_2^- in biochemical systems. This is particularly true for the system, where the oxidation to NO_2^- was originally observed, as ELSTNER and coworkers did find OH radicals in illuminated chloroplasts by the generation of ethylene from methional (3,6,7).

Materials and Methods: Hydroxylammonium chloride and sodium formate were used as obtained from Merck. Solutions were prepared with triply distilled and pyrolyzed water. The pH was adjusted with NaOH and no buffers were used. Nitrous oxide gas was purged from traces of oxygen by passing it through an Oxisorb column (Messer-Griesheim).

The pulse radiolysis set-up has been described before (8). New design features including an advanced photomultiplier control and direct evaluation of the data via transient recorder/digitizer (Datalab DL 905) and on-line computer link-up (Wang 2200 B) shall be published elsewhere (manuscript in preparation).

Results and Discussion: The transient spectra in neutral solutions of hydroxylammonium chloride (10^{-3} M) are shown in Figure 1. For better comparison the initial transients are shown at 20 μsec after the pulse. The different observation times for the end spectra are close to or coincide with a stable final absorption. Thus only in oxygenated solutions in the absence of formate (Figure 1c) an absorbing species is evident, which remains constant after 1 sec.

Figure 1 - Transient spectra of hydroxylamine

Based on the primary reactions of the radicals of water with the added solute sodium formate (10^{-2} M), respectively the gases nitrous oxide and oxygen (8), we can differentiate between three oxidizing conditions: in N_2O-saturated solutions only OH radicals (+ 10% reducing H atoms) are present, oxygenated solutions containing formate generate only O_2^-, whereas simply oxygenated solutions contain both OH and O_2^- radicals. The additional presence of chloride ions during pulse radiolysis of hydroxylamine can be neglected in neutral solutions. The oxidation of Cl^- by OH radicals is absolutely dependent on H^+ - the reaction proceeding by a third-order process (9):.

/2/ $\quad OH + Cl^- + H^+ \longrightarrow Cl + H_2O$

As can be seen, reaction of OH with NH_2OH (Figure 1a) results in an unstable transient species with an absorption maximum at 215 nm. This compound has previously been identified by SIMIC and HAYON as the NHOH radical,

being generated by the attack of OH with a rate constant of 9.5×10^9 M^{-1} sec^{-1} (10).

/3/ $NH_2OH + OH \longrightarrow NHOH + H_2O$

In anaerobic solutions this species decays by a close to second-order process (10), resulting in non-absorbing products:

/4/ $NHOH + NHOH \longrightarrow N_2 + 2\ H_2O$

In oxygenated solutions containing formate (Figure 1b) we observe only the initial absorption of O_2^- (λ_{max} = 250 nm), which decays with a close to theoretical second-order decay rate ($k_d = 2.6 \times 10^5$ M^{-2} sec^{-1} at pH 7) with no stable absorption peak being evident after more than 1 sec. Due to this absence of any accelerated decay of O_2^- in the presence of NH_2OH no rate constant could be determined.

Considerably different is the picture in oxygenated solutions without formate (Figure 1c). Here both the presence of two peaks at 215 and 250 nm in the 20 μsec-transient spectrum and a stable compound, also absorbing at 215 nm, are observed. The two species in the initial spectrum have been identified from the comparison with the previously discussed transients. Thus authentic NHOH radicals from the attack of OH show an absorbance ratio of $E_{240/215} = 0.37$, whereas in oxygenated solutions we obtain $E_{240/215} = 0.44$ (the ratio was determined for 240 nm in correlation to the peak at 215 nm as here the absorption of NHOH (Figure 1a) and NO_2^- (Figure 1c) shows the greatest difference). We can therefore safely assign the 215 nm-absorption in the 20 μsec transient spectrum to NHOH, with the slightly higher ratio due to the overlying absorption of O_2^- - this representing the second peak at 250 nm. The fact that O_2^- can be observed in oxygenated solutions even after 5 msec suggests that its reaction with the radical NHOH is also rather slow:

/5/ $NHOH + O_2^- \longrightarrow NO_2^- + H_2O$

The rate of O_2^--disappearance as compared to oxygenated solutions con-

taining formate indeed was only two orders of magnitude higher, which shows that the spontaneous dismutation of O_2^- is still in effect in competition to Reaction /5/. Due to mixed kinetics of this radical-radical reaction, no attempt was made to calculate an exact rate constant.

The formation of NO_2^- as final product absorbing at 215 nm in the above reaction was also established from the determination of the absorption ratio of $E_{240/215}$. The value of 0.06 was identical to authentic NO_2^-. The yield is rather low in comparison to the reactive radicals, as we calculated from a molar absorbtivity of 6.200 $M^{-1}cm^{-1}$ at 215 nm a final concentration of NO_2^- of 3.8×10^{-6} M (as compared to more than 10^{-5} M for each OH and O_2^-). Nitrogen dioxide as the product of a possible reaction of the NHOH radical with oxygen instead of O_2^-:

/6/ $\quad NHOH + O_2 \longrightarrow NO_2 + H_2O$

and absorbing at 400 nm (ε= 200 $M^{-1}cm^{-1}$; 11) could not be observed in oxygenated solutions of hydroxylamine.

The complete reaction sequence leading to NO_2^- has now to be envisioned as two successive reactions of both OH (Reaction /3/) and O_2^- (Reaction /5/). Based on the comparison of the overall stoichiometry:

/7/ $\quad NH_2OH + OH + O_2^- \longrightarrow NO_2^- + 2\ H_2O$

to the originally proposed Reaction /1/ (4), the determination of hydrogen peroxide should provide additional evidence for the cumulative attack of both OH and O_2^-. However, the H_2O_2 yield was only minimally higher than the theoretical value after one pulse of 2.5 - 3.2 krd (a G-value for the production of 0.7 molecules per 100 eV at a dose of 3.0 krd results in 2.1×10^{-6} M H_2O_2). The more complicated mechanism in alkaline solutions, where the overall yield of NO_2^- is decreased, shall be dealt with in a separate article (manuscript in preparation). This is of particular interest for the generation of NO_2^- during the autoxidation of adrenaline and

6-hydroxydopamine in alkaline solutions (unpublished results from our laboratory).

Conclusions: The method of pulse radiolysis rapidly generates substantial amounts of unstable oxygen radical species and thus allows the direct observation of their respective reactions with added solutes. However, the relatively high concentration of these radicals favors radical-radical interactions and therefore changes the overall mechanism. In biochemical systems with a low steady-state concentration of O_2^-, on the other hand, even slow reactions with solutes might be favored over the spontaneous decay of O_2^- via dismutation. While under such conditions formation of nitrite could conceivably still be due to an exclusive attack of O_2^-, one has to take into account the strong arguments against a closely analogous reaction (12,13), which incidently has been favored as the generation process for OH radicals - the so-called Haber-Weiss reaction (14):

/8/ $$O_2^- + H_2O_2 \longrightarrow OH + OH^- + O_2$$

Due to the low rate constant ($<10\ M^{-1}sec^{-1}$; CZAPSKI and ILAN, submitted for publication) the reaction is now considered an unlikely source of OH radicals. The same reasoning should apply to the reaction of O_2^- with NH_2OH in steady-state systems.

We assume, that OH radicals under biochemical conditions exist in an aggregated state with a H_2O_2/O_2^- complex and may be activated only in the presence of an adequate substrate. It is therefore likely, that even in systems where the generation of OH has not been conclusively proven by an independent method as the conversion of methional to ethylene (15) - though this assay has to be used with care (16) - hydroxylamine may present the first example of a specific test for the combined action of hydroxyl radicals and superoxide anions.

References:

(1) Elstner, E.F.; Heupel, A. and Vaklinova, S. (1970) Z. Pflanzenphysiol. 62, 184-200
(2) Elstner, E.F.; Heupel, A. and Vaklinova, S. (1970) Z. Pflanzenphysiol. 62, 173-183
(3) Elstner, E.F. and Konze, J.R. (1974) Ber. Dt. Botan. Ges. 87, 249-261
(4) Elstner, E.F.; Stoffer, C. and Heupel, A. (1975) Z. Naturforsch. 30c, 53-56
(5) Elstner, E.F. and Heupel, A. (1976) Anal. Biochem. 70, 621-623
(6) Elstner, E.F. and Konze, J.R. (1974) FEBS-Lett. 45, 18-21
(7) Elstner, E.F. and Konze, J.R. (1974) Z. Naturforsch. 29c, 710-716
(8) Bors, W.; Saran, M.; Michel, C.; Lengfelder, E.; Fuchs, C. and Spöttl, R. (1975) Int. J. Radiat. Biol. 28, 353-371
(9) Ward, J.F. and Myers, L.S. (1965) Radiat. Res. 26, 483-492
(10) Simic, M. and Hayon, E. (1971) J. Am. Chem. Soc. 93, 5982-5986
(11) Grätzel, M.; Henglein, A.; Lilie, J. and Beck, G. (1969) Ber. Bunsenges. Physik. Chem. 73, 646-650
(12) McClune, G.J. and Fee, J.A. (1976) FEBS-Lett. 67, 294-298
(13) Halliwell, B. (1976) FEBS-Lett. 72, 8-11
(14) Haber, F. and Weiss, J. (1934) Proc. Royal Soc., Ser. A 147, 332-351
(15) Beauchamp, C. and Fridovich, I. (1970) J. biol. Chem. 245, 4641-4646
(16) Bors, W.; Lengfelder, E.; Saran, M.; Fuchs, C. and Michel, C. (1976) Biochem. Biophys. Res. Comm. 70, 81-87