Hydrogen Isotope Effect on the Isomerization of Peroxynitrous Acid

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The kinetics of isomerization of deuterium and hydrogen oxoperoxonitrate, ONOOD and ONOOH, respectively, to trioxonitrate(1–), NO_3^-, and a deuteron or proton have been studied. Analysis of the pH-rate profiles result in a normal equilibrium isotope effect (K_H/K_D) of 3.3. The kinetic deuterium isotope effect for the isomerization reaction is 1.6 ± 0.2 between 5 and 55°. The activation enthalpies of isomerization of ONOOD and ONOOH are identical within the error of the measurement, 86.2 ± 0.5 and 86.5 ± 0.5 kJ mol^{-1}, respectively. A secondary kinetic isotope effect of 1.6 is compatible with a mechanism of isomerization where the terminal peroxide O-atom shifts to the N-atom.

1. Introduction. – Activated macrophages produce both superoxide and nitrogen monoxide [1], which react at a diffusion-controlled rate to form oxoperoxonitrate(1–) (peroxynitrite) [2]. This anion and its conjugate acid, O=NOOH, are potent oxidants [3] that react with and damage biomolecules [4][5]. Although oxoperoxonitrate(1–) is relatively stable in alkaline solution (pH $\geq$ 12), hydrogen oxoperoxonitrate (ONOOH) isomerizes to nitrate at a rate of 1.2 s^{-1}; its pK_a is 6.5 at low phosphate concentration [2]. ONOOH is generally more reactive than the anion, and may be mainly responsible for the cytotoxic reactions of oxoperoxonitrate(1–). The mechanism of its isomerization to nitrate is not known [6]. Information about reaction mechanisms can be obtained from isotope studies, where an atom in a reactant is replaced by an isotope, and the effect on rates of reaction and equilibrium constants is determined. Primary isotope effects, where the reaction involves a complete cleavage or formation of a bond to the labelled atom, are relatively large; secondary isotope effects, where the reacting bond is remote from the isotope, are smaller.

In the present study, the kinetic isotope effect for the isomerization of ONOOH was determined. We show that there is a secondary isotope effect on the decay of hydrogen oxoperoxonitrate over a wide pH range. The magnitude of the kinetic isotope effect is consistent with isomerization of ONOOH *via* intramolecular rearrangement.

2. Results. – The rates of isomerization of ONOOH and ONOOD are highly pH-dependent. The first-order rate constant calculated from the pH/pD profile for the decay of ONOOD is 1.35 times slower than that of ONOOH in the range of pH 5.4 to 8.1 as shown in *Fig. 1*. The pK_a values calculated from the pH/pD-rate profiles are 6.8 for ONOOH, the value reported earlier [2] for 25° and an ionic strength of μ = 0.1M, and 7.3 for ONOOD, respectively, which results in an equilibrium isotope effect of K_H/K_D = 3.3.

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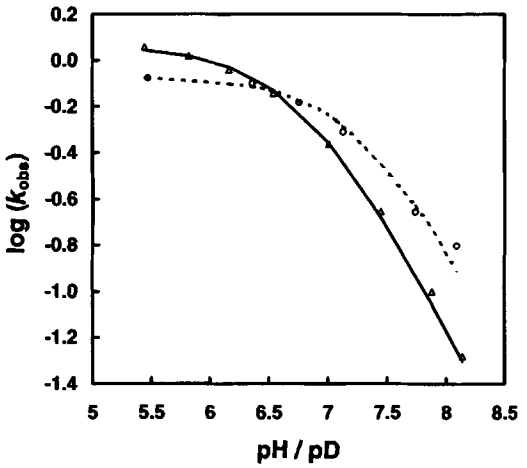


Fig. 1. *pH Dependence for the decay of ONOOH in H_2O* (triangles) *and ONOOD in 99% D_2O* (circles). Phosphate buffer, 0.1M; total concentration of oxoperoxonitrate(1–), 20 μM; $T = 25°$. The solid and the dashed line represent the fitted curves for the evaluation of the pK_as.

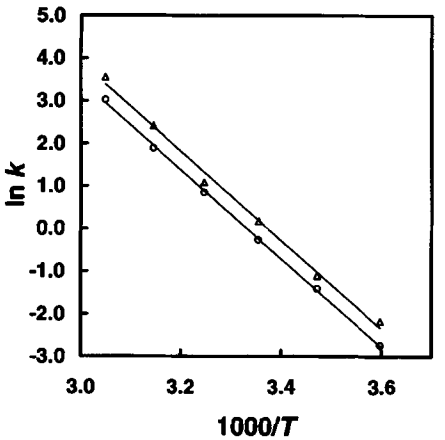


Fig. 2. Arrhenius *plots for the isomerization of ONOOH in H_2O* (triangles) *and ONOOD in 99% D_2O* (circles). Conditions: dihydrogen phosphate concentration, 0.1M; pH after mixing with the alkaline oxoperoxonitrate(1–) solution, 4.4; total concentration of oxoperoxonitrate(1–), 20 μM; temperature range, 5–55°.

The isomerization rate constants for ONOOH and ONOOD were also measured at several temperatures from 5 to 55°. The temperature dependences are shown in an *Arrhenius* plot (*Fig. 2*). The activation energy found for ONOOD (86.2 ± 0.5 kJ mol^{-1}) is identical to that of ONOOH (86.5 ± 0.5 kJ mol^{-1}) within the experimental error. Since the rate constants are different, the frequency factor, A, is the source of the difference in the activation parameters. We found $A_H = 1.8 \pm 0.5 \times 10^{15}$ for ONOOH and $A_D = 1.0 \pm 0.4 \times 10^{15}$ for ONOOD by extrapolation of the *Arrhenius* plots. The ratio A_H/A_D is 1.77, similar to the kinetic isotope effect k_H/k_D of 1.6 ± 0.2, over the tempera-

Table. *Temperature Dependence of the Deuterium Isotope Effect on the Rate of Isomerization*

Temperature [K]	k_H/k_D
278	1.77
288	1.37
298	1.55
308	1.27
318	1.68
328	1.71
Mean	1.6 ± 0.2

ture range investigated, as expected given the equal activation energies. The temperature-dependent rate constants and the corresponding k_H/k_D ratios are given in the *Table.*

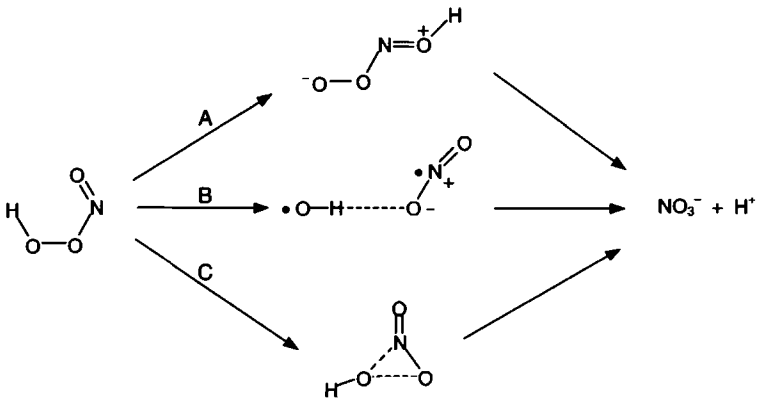
3. Discussion. – The equilibrium deuterium isotope effect of 3.3 for the ionization of ONOOH is in the range expected for an electrically neutral weak acid with a pK_a near 7 [7].

The kinetic isotope effect of 1.6 ± 0.2 found for the isomerization reaction is similar to an effect of 1.4 obtained earlier in our laboratory [8]. The effect is too small for a typical primary kinetic isotope effect which involves the cleavage or formation of a bond to the isotopic atom in the rate-determining step. The theoretical maximum value for a primary isotope effect for O–H bonds is 12, according to calculations that take into account changes in bond-stretching vibrations [7]. The theoretical maximum value for a secondary kinetic isotope effect for an O–H bond, *i.e.*, in a group adjacent to the center of reaction, is 2.02 [9]. Experimentally determined values are generally significantly smaller than the theoretical maxima for both primary and secondary kinetic isotope effects.

Three conditions have been described which reduce a primary isotope effect [10]. A bent transition state in the transfer of the H-atom, which is rarely the case except for intramolecular atom-shift reactions, leads to a very large decrease in the isotope effect. The second possibility for a decrease of the primary isotope effect is a large absolute value of the *Gibbs* energy change of the reaction. The ΔG° value for the ONOOH isomerization is $-167\ kJ\,mol^{-1}$ [3], which is not exceptionally high. A third condition which diminishes a primary isotope effect is the movement of a heavy atom together with the H-atom along the reaction coordinate. Here the reduced mass for the transition state stretching vibration mode of the H-bearing group is strongly determined by the heavy atom and not by the H- or D-atom [10]. The activation energies then should be quite similar for H and D, which is the case for ONOOH. However, primary C–H isotope effects smaller than 2 have rarely been reported, thus the observed effect of 1.6 for an O–H bond is more consistent with a secondary effect.

Two proposed mechanisms for the isomerization of ONOOH proceed through intermolecular H transfer with linear transition states. One of these proposals involves protonation of the oxo terminus of ONOOH [11] (*Scheme, Mechanism A*). This mechanism can be ruled out as such a protonation would likely be rate-limiting and have a primary isotope effect.

Scheme



Secondly, *Houk et al.* [12] have proposed O–O homolysis followed by *ca.* 180° turn and stabilization *via* H-bonding of the hydroxyl radical to nitrogen dioxide as a solvent-caged radical pair (*Scheme*, *Mechanism B*). Earlier we proposed an intramolecular rearrangement of the terminal O-atom to the N-atom (*Scheme*, *Mechanism C*) [6].

The observed secondary kinetic isotope effect of 1.6 is well below the theoretical maximal value of 2.02 estimated for α-OH [10]. Importantly, the activation energies for the isomerization reactions of ONOOH and ONOOD are equal, thus the difference in rates is not determined by differences in activation energies, but by some time-dependent function such as vibrational frequencies. These are higher in a OH moiety than in the corresponding ones of OD for all vibrational modes. It is thus likely that a bending vibration initiates the isomerization reaction, since these can be excited to a large extent even at room temperature.

Both processes, *Mechanisms B* and *C* in the *Scheme*, would be initiated by the excitation of the bending vibration of the OH moiety relative to the rather rigid O–N=O moiety. We now argue that homolysis is less likely than an intramolecular rearrangement.

Vibrational mode calculations for ONOOH performed with the program Gaussian94 [13] and visualized with MOLDEN [14] show that, upon thermal excitation, the OH group swings to a position over the plane defined by the O–N=O moiety, and into the vicinity of the N-atom consistent with the intramolecular rearrangement hypothesis.

The magnitude of the observed secondary deuterium isotope effect does not favor homolysis. Experimental values reported for C–CH cleavage [7] can be used for comparison, because the theoretical maxima for secondary OH and CH effects, 2.02 and 1.74 [9], respectively, are similar. The largest effect reported for a secondary isotope effect in a homolysis reaction involving C is 1.27, and the average is 1.05 [7]. A secondary isotope effect in an O–OH homolysis reaction is expected to be greater, but the value of 1.6 found for ONOOH would be at the upper limit.

Homolysis would be followed by rapid recombination of nitrogen dioxide with the hydroxyl radical, which is known to yield mainly, or only, ONOOH [15].

The isomerization of ONOOH as a function of pressure an activation volume of $1.7 \pm 1.0\ cm^3 mol^{-1}$ was determined, whereas homolytic reactions typically have activation volumes of *ca.* $10\ cm^3 mol^{-1}$ [2].

It is difficult to accept that a radical pair as shown in the *Scheme*, *Mechanism B*, could be stabilized by H-bonding when one considers that H-bonds to H_2O molecules must be broken as a result of its formation. *Bohle* and *Hansert* [16] argue that *Mechanism B* would result in complete oxygen isotope retention in their ^{18}O-scrambling experiments, which they did not observe. If the hydroxyl and nitrogen dioxide radicals were to become separated, complete ^{18}O-scrambling would occur, which was not found either [16].

We conclude, therefore, that the isomerization probably proceeds through intramolecular rearrangement of the terminal O-atom of the peroxo group to the N-atom, as shown in the *Scheme*, *Mechanism C*. The protonation or the addition of a *Lewis* acid [2] facilitates this process by withdrawal of negative charge density from the peroxo terminal, thus making the nitrogen more electrophilic and reducing the repulsion between the nitrogen lone pair electrons and the high negative charge density on the terminal peroxo O-atom.

4. Experimental. – Deuterium oxide (99%) and sodium deuteroxide were obtained from *Aldrich Chemicals* and *Cambridge Isotope Laboratories*. Sodium azide was from *EM Science*. Potassium superoxide was from *Aldrich* and *Fluka*. Nitrogen monoxide was obtained from *Linde*.

Sodium oxoperoxonitrate (ONOONa) was synthesized *via* reaction of NaN_3 with O_3 [17]. ONOOK was produced from the reaction of solid KO_2 with gaseous NO [18]. For solns. in deuterated environment, the azide-ozone reaction was carried out in 99% D_2O, or the ONOOK obtained from the solid-gas reaction was dissolved in 0.01M NaOD soln. in 99% D_2O. Fresh hydroxide solns. were prepared daily in order to avoid carbonate accumulation, which can affect the kinetics of oxoperoxonitrate(1 –) reactions by release of CO_2 upon acidification [19].

The pH of the solns. was adjusted with phosphoric acid and phosphate salts, and ionic strength was kept at 0.1M by adjusting the concentration of the buffers. Deionized *Milli-Q* water and 99% D_2O were used for preparing solns. of ONOOH and ONOOD, respectively. Spectra were recorded on *Beckman DU-7HS* and *Kontron Uvikon 820* spectrophotometers.

The kinetics of the reactions were followed with an *On-Line Instrument Systems, Inc.* stopped-flow instrument equipped with an *OLIS RSM 1000* rapid scanning monochromator and with an *Applied Photophysics SX17MV* single-wavelength stopped-flow instrument. With the *OLIS* instrument, the decay of ONOOH and ONOOD was monitored from 250 to 350 nm, and the data were analyzed with *OLIS-RSM 1000* global fit software. With the *Applied Photophysics* apparatus, kinetic traces were taken at 302 nm at neutral and alkaline pH and at 260 nm in the acidic range, and data were analyzed with the *SX17MV* operating software. Traces from six to nine experiments were averaged to obtain each observed rate constant. At pH < 6, the total concentration of oxoperoxonitrate(1 –) was 100 μM, but at higher pH the total concentration was limited to 20 μM in order to reduce the extent of the reaction between acid and anion [2].

Temp. were maintained to $\pm 1°$ with a *VWR* model *1160* circulating water bath in the *OLIS* instrument and by a *Haake* thermostat in the *Applied Photophysics* spectrometer. For temp. dependence experiments, the *Applied Photophysics* instrument was equipped with an extra thermocouple probe which was attached directly to the observation cell inside the thermostat mantle, since readings from the built-in probe were found to be incorrect at temp. below 15° and above 30°. The thermocouple probe was calibrated with boiling water, the temp. of which was corrected for the ambient pressure, and an ice-water mixture.

We thank *Andreas Eggenschwiler* for the computer modelling of the ONOOH vibrations. This work was supported by the *National Institutes of Health* (GM 48829), the *Council for Tobacco Research-USA, Inc.*, ETH-Zurich, and the *Swiss National Science Foundation*.

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Received April 9, 1998