

## Role of Conformation of Peroxynitrite Anion (ONOO<sup>-</sup>) in Its Stability and Toxicity

Jyh-Hsin M. Tsai,<sup>†,‡</sup> Joseph G. Harrison,<sup>†</sup> James C. Martin,<sup>†</sup> Tracy P. Hamilton,<sup>§</sup> Mark van der Woerd,<sup>⊥</sup> Michael J. Jablonsky,<sup>||</sup> and Joseph S. Beckman\*<sup>†</sup>

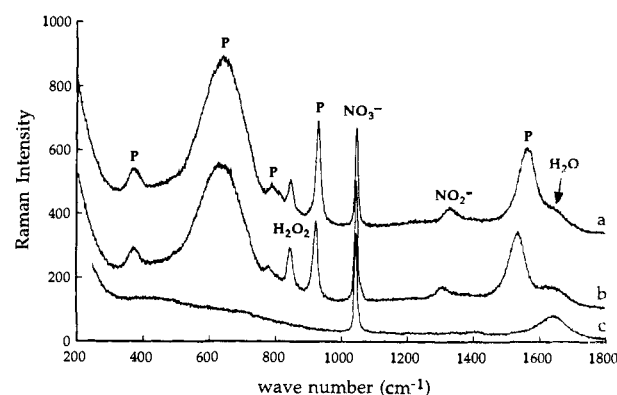
Departments of Anesthesiology, Physics, and Chemistry  
The Center for Macromolecular Crystallography, and  
Comprehensive Cancer Center NMR Core Facility  
The University of Alabama at Birmingham  
Birmingham, Alabama 35233

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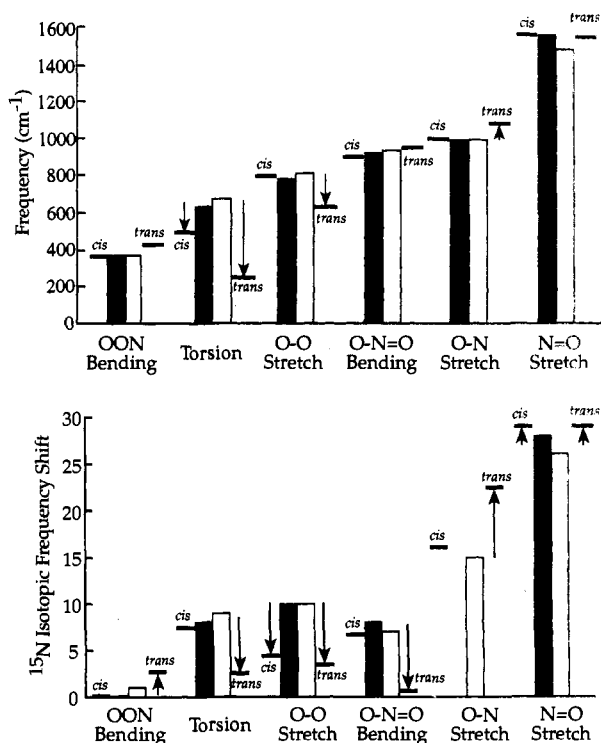
Peroxynitrite anion (ONOO<sup>-</sup>), the product of superoxide reacting with nitric oxide, is a strong and relatively long-lived oxidant produced by macrophages and neutrophils.<sup>1</sup> It has been implicated in pulmonary edema, myocardial ischemia, and stroke.<sup>2–4</sup> Peroxynitrite formed by ultraviolet irradiation of nitrate in the Martian soil may account for the initially encouraging indications of life during the Viking missions.<sup>5</sup> A major factor contributing to peroxynitrite's toxicity is its stability as an anion, even though peroxynitrite is 36 kcal·mol<sup>-1</sup> higher in energy than its isomer, nitrate.<sup>6</sup> Peroxynitrite remains in alkaline solution for days and is indefinitely stable in alkali nitrates exposed to ultraviolet light.<sup>7</sup>

Peroxynitrite anion can potentially exist in two planar geometries, the *cis* and *trans* isomers, but only one isomer is observed by Raman spectroscopy. Of the 12 possible vibrational modes (six for each isomer), the Raman spectrum of peroxynitrite exhibited only five bands in alkaline solution, with a sixth band apparently hidden beneath a strong nitrate band centered at 1047 cm<sup>-1</sup> (Figure 1). In ultraviolet-irradiated potassium nitrate crystals, the peak positions of peroxynitrite were similar to those in the solution spectrum and the sixth band was observable<sup>8</sup> at 1000 cm<sup>-1</sup>. Thus, only one peroxynitrite isomer was observed in both the alkaline solution and the solid state.

*Ab initio* quantum chemical calculations based upon the *cis* isomer in the gas phase predicted the observed spectra more accurately than calculations based upon the *trans* isomer (Figure 2a). Furthermore, the frequency shifts caused by <sup>15</sup>N-substituted peroxynitrite were more accurately reflected by the *cis* conformation (Figure 2b). The calculations utilized the 6-311+G\* basis set<sup>9</sup> with electron correlation taken into account by the coupled cluster singles and doubles method (CCSD)<sup>11</sup> which predicted that the *cis* isomer is also approximately 3 kcal·mol<sup>-1</sup>



**Figure 1.** Raman spectra of 800 mM (a) O<sup>14</sup>NOO<sup>-</sup> and (b) O<sup>15</sup>NOO<sup>-</sup> in aqueous alkaline solutions. (c) The background spectrum was determined after peroxynitrite was decomposed with hydrochloric acid. Peroxynitrite anion was synthesized in a quenched-flow reactor and concentrated by freeze fractionation.<sup>10</sup> Raman spectra were recorded with the 488-nm line from an Ar<sup>+</sup> ion laser with output power levels of 500–600 mW and with a scan rate of 1 cm<sup>-1</sup>·s<sup>-1</sup> and a resolution of 5 cm<sup>-1</sup>.



**Figure 2.** (a, top) Quantum mechanical predictions of the vibrational spectra for *cis*- and *trans*-peroxynitrite anion (horizontal lines) compared to the observed Raman spectrum for (<sup>14</sup>N)peroxynitrite in solution (solid bars) and the solid state (open bars). (b, bottom) The frequency shifts of <sup>15</sup>N-substituted peroxynitrite relative to (<sup>14</sup>N)peroxynitrite predicted by quantum mechanical calculations compared to observed shifts.

more stable than the *trans* isomer. The greater stability of the *cis* isomer can be rationalized by the partial bonding character of the highest occupied molecular orbital (HOMO) between the two terminal oxygens (Figure 3), whereas such interactions are absent between the two terminal oxygens in the *trans* isomer.

Generally, Raman peaks are narrow, such as those observed for the nitrate peak at 1047 cm<sup>-1</sup> in Figure 1. All of the peroxynitrite peaks in the solution spectrum are broadened by strong interactions with water, and the torsional band is exceptionally broad and strong. Spectra were obtained with 488- and 514.5-nm laser excitation wavelengths to confirm that these are true Raman bands rather than fluorescent artifacts. The strength of the torsional band is in part due to the large change

\* Author to whom correspondence should be addressed. Phone (205) 934-5422. FAX (205) 934-7437.

<sup>†</sup> Department of Anesthesiology.

<sup>‡</sup> Department of Physics.

<sup>§</sup> Department of Chemistry.

<sup>⊥</sup> The Center for Macromolecular Crystallography.

<sup>||</sup> Comprehensive Cancer Center NMR Core Facility.

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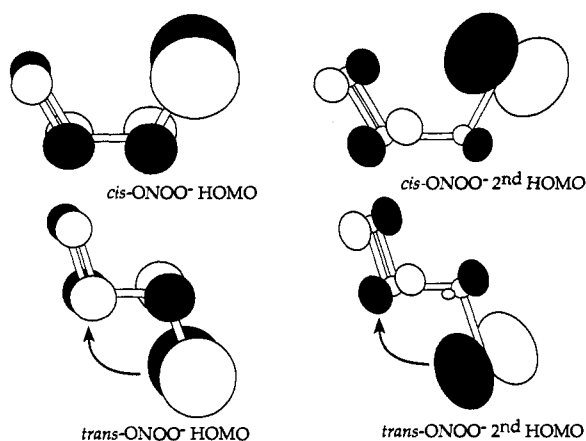
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**Figure 3.** Highest and second-highest occupied molecular orbitals (HOMO and 2nd HOMO) for *cis*- and *trans*-peroxynitrite anions. Positive and negative phases of the wave function are indicated in white and black. The arrow indicates how the terminal peroxide oxygen can directly attack the nitrogen on *trans*-peroxynitrite to form nitrate. No such attack is possible for the *cis* isomer.

in polarizability when peroxynitrite twists out of the planar conformation, thereby decreasing bonding. Changes in the induced dipole on adjacent water molecules also contribute to the broadening of the torsional band because the band narrowed when water was replaced with less polar solvents. The torsional band was also much narrower and weaker in the solid-state spectrum. The frequency predictions were poorest for the torsional band, which we attribute to strong interactions with water in the aqueous spectra and with lattice interactions in the solid state that were not taken into account by the gas-phase calculations. However, the frequency shift of the torsional band of  $^{15}\text{N}$ -substituted peroxynitrite was accurately predicted by the quantum mechanical calculations based upon the *cis* conformation (Figure 2b).

Because the negative charge in the *cis* conformation is partially delocalized over the entire peroxynitrite molecule, a weak partial bond is formed between the terminal oxygens with the strength of a typical hydrogen bond (Figure 3). This stabilization contributes to peroxynitrite having the lowest  $\text{p}K_a$ , 6.8, of any peroxide-containing compound<sup>12</sup> and partially locks peroxynitrite into the *cis* conformation by limiting the rotation of the terminal oxygen around the  $\text{OO}-\text{NO}$  bond. Calculations indicate that a 21–24  $\text{kcal}\cdot\text{mol}^{-1}$  barrier limits isomerization between the *cis* and *trans* anions. The terminal peroxide oxygen cannot directly attack the nitrogen to form nitrate, because contracting of the  $\text{O}-\text{O}-\text{N}$  bond angle produces a strong repulsion between the terminal peroxide oxygen and the two oxygens bound to the nitrogen. In contrast, the terminal oxygen in the *trans* isomer

can directly attack the nitrogen by contraction of the  $\text{N}-\text{O}-\text{O}$  bond angle coupled with a slight lengthening of the  $\text{O}-\text{O}$  bond, allowing *trans*-peroxynitrite to directly rearrange to nitrate (Figure 3).

The 21–24  $\text{kcal}\cdot\text{mol}^{-1}$  barrier is too high for the isomerization to occur at a significant rate at physiological temperatures, but protonation to form peroxynitrous acid ( $\text{ONOOH}$ ), the conjugate acid of peroxynitrite, substantially lowers the barrier for isomerization of the acid to  $\sim 10$ –12  $\text{kcal}\cdot\text{mol}^{-1}$  in the gas phase. Peroxynitrous acid is also more stable in the *cis* form by 1–2  $\text{kcal}\cdot\text{mol}^{-1}$ .<sup>13–15</sup> Because the energy difference between the *trans*-peroxynitrite anion and the acid is approximately 2  $\text{kcal}\cdot\text{mol}^{-1}$  larger than the difference between the *cis*-peroxynitrite anion and the acid, the  $\text{p}K_a$  of *trans*-peroxynitrite should be roughly 1–1.5 pH units higher than the *cis* conformation.

We have previously shown that the product yields from peroxynitrite are strongly dependent upon pH and exhibit two distinct  $\text{p}K_a$  values of 6.8 and at approximately 8.0, based upon what type of substrate is attacked.<sup>16</sup> For example, peroxynitrite directly attacks sulfhydryls with an apparent  $\text{p}K_a$  of 6.8.<sup>17</sup> The hydroxyl radical-like reactivity appears to be derived from a vibrationally excited state derived from *trans*-peroxynitrous acid<sup>16,18</sup> which decreases at alkaline pH with an apparent  $\text{p}K_a$  of 8.0.<sup>10,16</sup> Nitration of phenolics catalyzed by superoxide dismutase is maximal at pH 7.5 and has two  $\text{p}K_a$  values of 6.8 and 7.9.<sup>19</sup> The stability of the *cis* conformation makes peroxynitrite far more toxic by allowing it to diffuse long distances on a cellular scale and results in unexpected complex oxidative chemistry in the physiological pH range due to isomerization between the *cis* and *trans* conformations.<sup>16–19</sup>

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