On the acidity and reactivity of HNO in aqueous solution and biological systems

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The gas phase and aqueous thermochemistry and reactivity of nitroxyl (nitrosyl hydride, HNO) were elucidated with multiconfigurational self-consistent field and hybrid density functional theory calculations and continuum solvation methods. The pK_a of HNO is predicted to be 7.2 ± 1.0, considerably different from the value of 4.7 reported from pulse radiolysis experiments. The ground-state triplet nature of NO^- affects the rates of acid-base chemistry of the HNO/NO^- couple. HNO is highly reactive toward dimerization and addition of soft nucleophiles but is predicted to undergo negligible hydration ($K_{eq} = 6.9 \times 10^{-5}$). HNO is predicted to exist as a discrete species in solution and is a viable participant in the chemical biology of nitric oxide and derivatives.

The discoveries of nitric oxide (NO) biosynthesis in mammalian cells and the diverse biological activity associated with NO and NO-derived species (1) have brought intense interest in the physiological chemistry of nitrogen oxides. The chemistry of NO and its biologically accessible oxidized congeners nitrogen dioxide (NO_2), nitrite (NO_2^-), peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3), and nitrate (NO_3^-) is fairly well established (2). By contrast, the reduced congeners such as nitroxyl (HNO) and its conjugate base (NO^-) are less well understood, and consequently their role in biology is not clear. The importance of HNO or NO^- in biology has often been neglected or dismissed, in part because NO metabolism is thought to be primarily oxidative in nature (3), and because HNO is thought to be only metastable (3), a strong acid (4), and to dimerize readily (5). NO^- is known to react rapidly and irreversibly with NO (6), making the examination of NO^- in the presence of NO difficult. Additionally, HNO might be expected to be electrophilic, and hydration under physiological conditions would serve to attenuate its aqueous reactivity.

Nitroxyl (HNO), or its conjugate base, NO^-, is known to be formed under physiological conditions; for example, oxidation of *N*-hydroxy-L-arginine (an intermediate in NO biosynthesis) (7), reaction of *S*-nitrosothiols with thiols (8, 9), nitric oxide synthase (10–12), and even direct reduction of NO by mitochondrial cytochrome *c* (13), may all generate HNO. Nitroxyl has been generated via the interaction of NO with manganese superoxide dismutase (14) and with ubiquinol (15). HNO has biological activity; it can act as a potent cytotoxic agent that causes double-stranded breaks in DNA, depletion of cellular glutathione (16), as well as elicitation of smooth muscle relaxation (17). HNO has been found to be a potent inhibitor of thiol-containing enzymes (18, 19) and attenuates the activity of the NMDA receptor via thiol modification, thus providing neuroprotection (20).

Much of the fundamental biological chemistry associated with HNO is unknown, aside from the rate constant for dimerization ($2–8 \times 10^9\ M^{-1}\ s^{-1}$) (5). The pK_a of HNO has been reported to be 4.7, as determined by pulse radiolysis studies (4), indicating that NO^- will be the near-exclusive species present at physiological pH. Subsequent studies by Seddon *et al.* demonstrated the temperature and pH dependence of decay of both NO^- and higher-order adducts $N_2O_2^-$ and $N_3O_3^-$ (6). NO^- is a typical nucleophile, yet several studies find that HNO generated at physiological pH reacts readily as an electrophile, particularly with thiols, to yield *N*-hydroxysulfenamide intermediates (9, 21), implying a higher pK_a.

NO^- and NO react rapidly and irreversibly to form $N_2O_2^-$, a reactive radical anion (6). Nevertheless, at likely physiological concentrations of NO ($<1\ \mu M$) (22), the rate of disappearance of NO^- and NO via bimolecular reaction will be slow compared with that of other biological processes.

The generation of HNO has been observed via the decomposition of Piloty's acid (benzenesulfohydroxamic acid) at pH 8 (23). However, the pK_a value of 4.7 for HNO would preclude the existence of appreciable concentrations of HNO under these conditions. The unfavorable free energy of protonation at neutral pH (3.2 kcal/mol) would contribute to low reactivity.

We have used quantum mechanical calculations that predict the fundamental chemical properties of HNO in solution and herein report computational results that establish the aqueous thermochemistry of nitroxyl and its reactivity toward species present under physiological conditions. The significant concentration of HNO now predicted at physiological pH, relative to NO^-, and the high reactivity toward thiols open new possibilities for the involvement of nitroxyl in biological mechanisms.

Energetics of Singlet and Triplet HNO and NO^-

The lowest triplet ($^3A''$) excited state of HNO has been determined spectroscopically to lie 18 kcal/mol above the singlet ($^1A'$) ground state (24). QCISD(T) and MP2 calculations reported by Brauman *et al.* (25, 26) agree well (Table 1).

It is not generally known whether the isomeric HON species is biologically accessible. Spectroscopic and theoretical studies (27–31) and our own calculations place the energy of this triplet ground-state species at 20–23 kcal/mol above the HNO singlet ground state. Therefore, HON is unlikely to be a participant in the physiological chemistry of HNO.

NO^- Is a Ground-State Triplet, Isoelectronic with Dioxygen

Experimental measurements place the singlet state of NO^- at ≈17 kcal/mol above the ground state (32, 33). We have optimized the singlet and triplet states of NO^- with complete active space self-consistent field calculations (34). An (8e, 6o) active space was used, corresponding to full configuration interaction in the 2p valence space. The singlet-triplet (S-T) energy gap,

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Abbreviation: PCM, Polarizable Continuum Model.

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Table 1. Calculated and experimental singlet-triplet gaps for NO^-, O_2, and HNO

Species	*E*(singlet) − *E*(triplet), kcal/mol				
	CASSCF	CASMP2	QCISD(T)	MP2	Experiment
NO^-	20.5*	20.9*	18.0†	18.0‡	17.2§
O_2	20.3*	22.5*			22–24¶
HNO			−18.0†	−18.1‡	−17.9‖

*This study.
†Ref. 25.
‡Ref. 26.
§Ref. 32.
¶Ref. 36.
‖Ref. 24.

calculated with inclusion of MP2 correction to the CASSCF energy (CASMP2) (35), is provided below in Table 1 and compared with values computed for O_2 at the same level of theory. The S-T gap of NO^- is predicted to be 21 kcal/mol, suggesting a value slightly higher than the experimental estimates. The calculated value for the isoelectronic O_2, 23 kcal/mol, by using this same method, is in excellent agreement with experiment (36).

The pK_a of HNO

Is $^3NO^-$ protonated at pH 7? Geometry optimizations and harmonic frequency analyses of a series of organic and inorganic acids and their conjugate bases were performed with hybrid density functional theory (B3LYP/6–311+G*) (37). Aqueous solvation energies were determined by using the Polarizable Continuum Model (PCM) of Tomasi and coworkers (38, 39). These values are given in Table 2. The gas-phase deprotonation energies plus aqueous solvation energy differences between acid and conjugate base show a good linear correlation with experimental pK_a values (40, 41). The relationship $pK_a = 0.549\ (PA_{calc,\ PCM}) - 139.8$ was obtained, with a correlation coefficient of 0.95 and standard deviation of 1.0 pK_a units. Fig. 1 shows a plot of the predicted versus experimental pK_a values.

By using this correlation, a calculated pK_a of 7.2 ± 1.0 was determined for the $HNO + H_2O/H_3O^+ + {}^3NO^-$ equilibrium, indicating that about 50% of HNO should exist at equilibrium at physiological pH. This value is substantially higher than the pulse radiolytic estimate of 4.7, and more consistent with recent experimental observations of Wong *et al.* and of Shoeman *et al.*, which suggest a substantial concentration of the nondeprotonated form (9, 19). In the original study by Grätzel *et al.* (4), difficulty in obtaining an accurate NO^- concentration measurement directly after the radiolysis pulse

Table 2. Calculated gas-phase deprotonation energies (PA), solvation energies, and experimental and calculated pK_a values

Acid	pK_a (exp)*	PA (calc, gas),† kcal/mol	$\Delta\Delta G_{solvation}$,‡ kcal/mol	pK_a, (calc)§
Trifluoroacetic acid	0.2	314.6	60.9	−0.5
Trichloroacetic acid	0.7	312.7	59.8	−1.0
Nitrous acid	3.3	331.5	75.8	0.6
Dinitromethane	3.6	314.9	53.4	3.8
Formic acid	3.8	335.6	74.8	3.4
Benzoic acid	4.2	333.8	67.9	6.2
Acrylic acid	4.3	337	72.2	5.6
Hydrazoic acid	4.6	336.2	72.6	4.9
Acetic acid	4.8	340.6	74.3	6.4
Dimethyl malonate	9	336.1	58.5	12.6
Hydrogen cyanide	9.2	346.4	79.6	6.7
Phenol	10.0	341.5	70.6	8.9
Nitromethane	10.2	352.2	72.1	14.0
Methanethiol	10.3	352	75.2	12.2
Ethanethiol	10.6	350.4	71.0	13.6
Dicyanomethane	11.2	328	55.2	10.0
Trichloroethanol	12.2	345.7	72.9	10.0
Trifluoroethanol	12.4	352	71.2	14.4
Propargyl alcohol	13.6	362.9	84.1	13.4
Allyl alcohol	15.5	365.8	84.1	14.9
Methanol	15.5	373.9	92.3	14.8
Water	15.7	384.7	102.8	15.0
Ethanol	15.9	371.0	88.6	15.2
Isopropanol	17.1	369.2	85.6	15.9
t-Butanol	18	368.5	83.2	16.8
Acetone	19.3	366.8	73.1	21.5
Acetonitrile	25	370.2	70.1	25.0
Methyl acetate	25.4	370.5	66.8	27.0
Dimethyl sulfone	33	365.7	56.5	30.0
Dimethyl sulfoxide	33.5	374.6	63.2	31.2
HNO		349.6	81.9	7.2

exp, experimental; calc, calculated.
*Literature data taken from refs. 40 and 41.
†B3LYP/6-311+G* + ZPE.
‡PCM-B3LYP/6-311+G*//B3LYP/6-311+G* + ZPE.
§Obtained from linear regression plot, $pK_a = 0.549\ (PA_{calc,PCM}) - 139.8$.

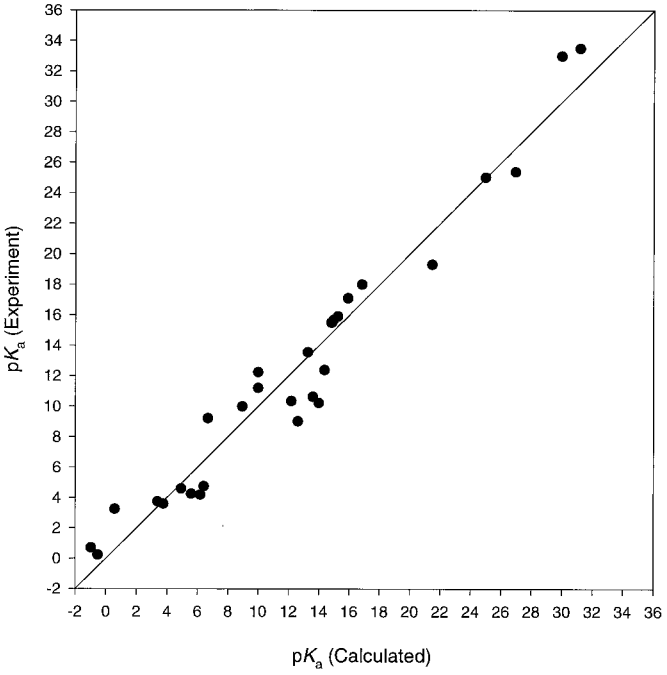

Fig. 1. Plot of experimental versus calculated pK_a. Data are listed in Table 2.

was noted, perhaps because of the generation of both singlet and triplet nitroxyl, and complicating equilibria between NO^- and its NO adducts, $N_2O_2^-$ and $N_3O_3^-$, under the high relative concentrations of NO used in the experimental conditions.

Singlet nitroxyl anion ($^1NO^-$) is predicted to possess an enormous pK_a value of 18.6 (obtained from the CASMP2 singlet-triplet gap of NO^- and the corresponding solvation energies). However, given the electronic similarity of NO^- and O_2, and short lifetime of singlet oxygen in aqueous solution ($\approx 1 \times 10^{-6}$ s) (36), any NO^- present in equilibrium with HNO in aqueous solution should exist in its triplet state. Proton transfer in this system should be slowed substantially relative to typical proton transfers involving conservation of spin. It has been shown by Brauman and coworkers for $^3NO^-$ in the gas phase (25, 26) that the process of proton transfer involving spin state interconversion is slowed by as much as 10^7, as compared with spin-conserving proton transfers.

Donald *et al.* originally suggested that the triplet forms of HNO should possess enhanced acidity versus the singlet state (42). We predict that the excited triplet state of HNO is an extremely potent acid in aqueous solution, with a pK_a of about -2 based on the spectroscopically determined singlet-triplet gap of HNO.

The Hydration K_{eq} of HNO

Because about 50% of HNO is undissociated at physiological pH, the nitroso group might be expected to hydrate, as do many aldehydes (43). We have compared the hydration of HNO with that of aldehydes and ketones. To calibrate our calculations and obtain a quantitative value for the hydration K_{eq} of HNO, the geometries of a series of carbonyl compounds and their respective hydrates were optimized at the B3LYP/6–311 + G* level. Solvation calculations using the PCM formalism were used to model aqueous solvation. Table 3 lists computed hydration energies and experimental values of K_{eq}. A relation of $\log K_{eq} = -0.56\ (\Delta E_{rxn,\ PCM}) + 0.32$ was obtained, with a correlation coefficient of 0.9 and standard deviation in $\log K_{eq}$ of 0.73. A plot of experimental versus calculated hydration equilibria is provided in Fig. 2.

Table 3. Calculated gas- and solution-phase energetics of hydrate formation [RCHO + $H_2O \rightarrow RCH(OH)_2$] and calculated experimental hydration equilibrium constants

Compound	K_{eq} (exp)*	ΔE_{rxn}, gas phase, kcal/mol†	ΔE_{rxn}, PCM, kcal/mol‡	K_{eq} (calc)§
$C_6H_5COCH_3$	9.3×10^{-6}	+2.3	+8.5	3.6×10^{-5}
$(CH_3)_2CO$	1.4×10^{-3}	−0.4	+4.4	7.2×10^{-3}
C_6H_5CHO	8×10^{-3}	+1.1	+5.2	2.6×10^{-3}
$(CH_3)_3CCHO$	2.3×10^{-1}	−2.0	+1.2	4.4×10^{-1}
CH_3CHO	1.06	−3.4	−0.3	3.08
CCl_3CHO	3×10^3	−5.3	−1.3	1.1×10^2
H_2CO	2.3×10^3	−7.5	−5.2	1.7×10^3
CF_3CHO	2.9×10^4	−9.6	−9.2	3.0×10^5
HNO		+7.6	+8.0	6.9×10^{-5}

exp, experimental; calc, calculated.
*Data taken from refs. 44–46.
†B3LYP/6-311+G* + ZPE.
‡PCM-B3LYP/6-311+G*//B3LYP/6-311+G*+ZPE.
§From linear regression of calculated ΔE_{rxn} in solution (column 4) versus experimental K_{eq} (column 2); $\log K_{eq} = -0.56\ (\Delta E_{rxn,PCM}) + 0.32$.

From this correlation, the hydration of HNO is predicted to be highly unfavorable, with a K_{eq} value of 6.9×10^{-5}. HNO resembles acetophenone in its reluctance to hydrate, in stark contrast to the analogous parent carbonyl species, formaldehyde ($K_{eq} = 2.3 \times 10^3$). Essentially all HNO exists in solution as such and does not hydrate to any significant extent. Repulsion between lone pairs on nitrogen and the two adjacent oxygens in the hydrate gives rise to the high energy of this hydrated species.

Dimerization and Reactivity Toward Nucleophiles

The thermodynamics of HNO dimerization and reactions with methanethiol, methylamine, and methanol were predicted at the B3LYP/6–311+G* level of theory. The results are shown in Fig. 3. HNO is predicted to be relatively inert to addition by

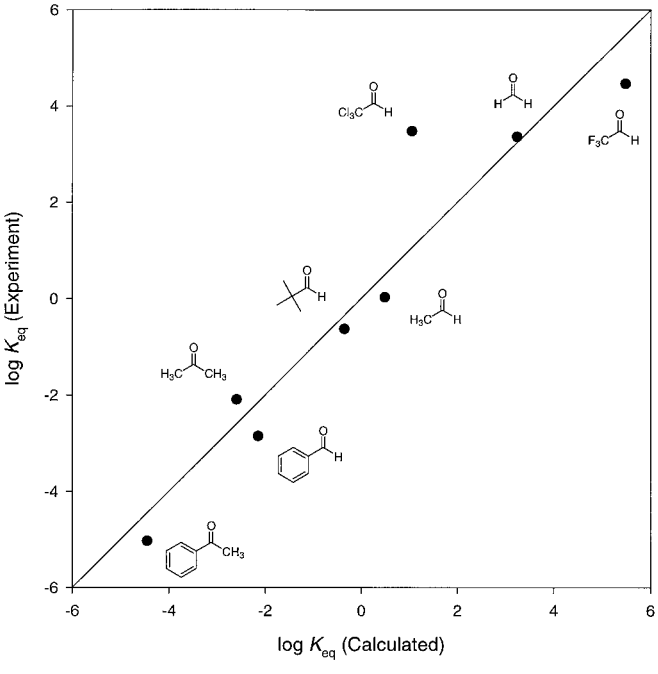

Fig. 2. Plot of calculated versus experimental hydration equilibria for aldehydes and ketones in Table 3. PCM-B3LYP/6–311+G*+ zero point energy.

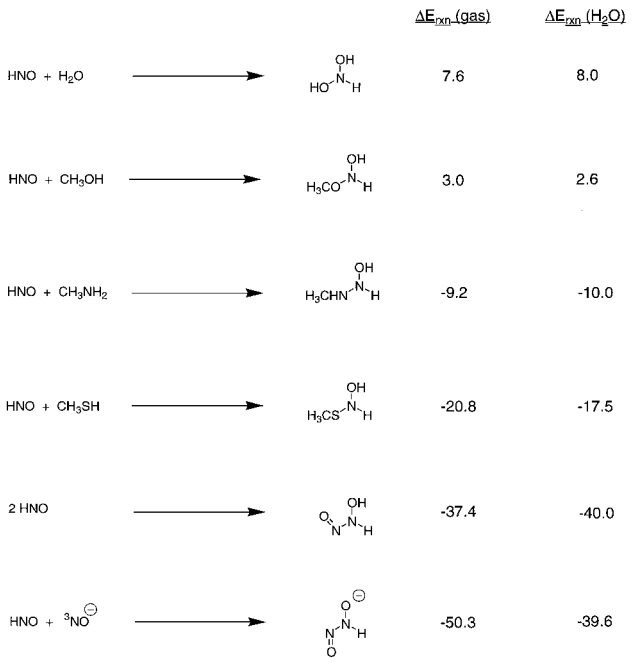

Fig. 3. Energies of reaction of HNO with nucleophiles and for dimerization (kcal/mol), in the gas phase [B3LYP/6–311+G*+ zero point energy (ZPE)] and in solution (PCM-B3LYP/6–311+G*//B3LYP/6–311+G*+ZPE).

oxygen-based nucleophiles, but reactions with amines and thiols are highly favorable in either the gas phase or solution. The latter is especially important, because nucleophilic addition to HNO is proposed to occur in the degradation of *S*-nitrosothiols in the presence of added thiol, to yield the corresponding sulfinamide and ultimately, NH_3 (9). The mechanism of aldehyde dehydrogenase inhibition is also thought to involve attack of the active-site sulfhydryl group at nitrogen of HNO (18, 19).

At high local concentrations of HNO, dimerization will provide a competing pathway for the HNO degradation, analogous to the dimerization of aliphatic *C*-nitroso compounds (47). HNO dimerization is rapid ($k_2 = 2–8 \times 10^9\ M^{-1}\ s^{-1}$) (5) and strongly thermodynamically favored, predicted to occur with an energy of reaction of −37 kcal/mol in the gas phase and −40 kcal/mol in aqueous solution (Fig. 3). This irreversible process leads ultimately to a molecule of N_2O and water, the mechanism of which has been studied in detail by *ab initio* and molecular dynamics methods (48, 49). Our prediction of a pK_a of about 7 means that in neutral and slightly acidic aqueous solutions, both HNO and NO^- will be present. The reaction between HNO and $^3NO^-$ is predicted to be highly thermodynamically favorable ($\Delta E_{rxn} = -40$ kcal/mol; Fig. 3). Once again, however, the rate of this spin-forbidden process will be slowed, analogous to the proton transfer reactions discussed above.

Conclusions

HNO is predicted to be stable in aqueous solution and only a weak acid. HNO reacts exothermically with soft nucleophiles such as amines and thiols but is relatively inert to oxygen-based nucleophiles. HNO is a highly reactive but selective electrophile, whereas NO is essentially inert as an electrophile. HNO joins NO and its oxidized congeners as a vital player on the biological stage. In light of the new pK_a values, we now provide for singlet and triplet NO^-, the redox potentials of these species must now be reassessed. The reduction potentials of −0.35 V and +0.39 V for the $NO/^1NO^-$ and $NO/^3NO^-$ couples, as estimated by Stanbury (50), used the pulse radiolysis data of Grätzel *et al.* (4) and assumed this pK_a value corresponded to singlet NO^-. These reduction potentials should be reevaluated, and further details of the chemical biology of HNO and its derivatives warrant additional investigation.

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