



Nitroxygenation of quercetin by HNO



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ABSTRACT

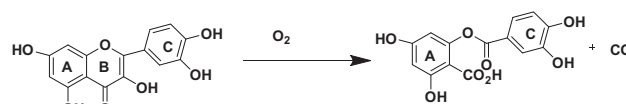
The flavonol quercetin undergoes both enzymatic and non-enzymatic reactions with nitroxyl (HNO/NO^-), similar to analogous reactions with dioxygen, but in which N is regioselectively found in the ring-cleaved product. Here we report on kinetic and thermodynamic analysis of the non-enzymatic nitroxygenation reaction in water, which is orders of magnitude faster than the comparable dioxygenation. The second order rate constants were determined from variable temperature reactions, which allowed determination of the reaction activation enthalpy ($\Delta H^\ddagger = 9.4 \text{ kcal/mol}$), entropy ($\Delta S^\ddagger = -8.3 \text{ cal/mol K}$), and free energy ($\Delta G^\ddagger = 11.8 \text{ kcal/mol}$). The determined standard state energy (ΔG°) and activation free energy, as well as the low entropic energy of reaction, are consistent with a proposed single electron transfer (SET) rate determining step.

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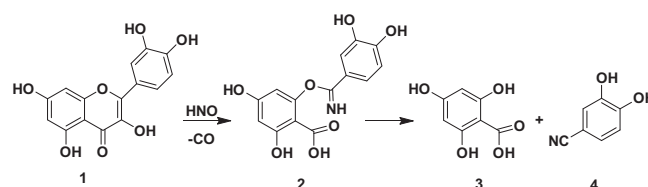
Quercetin and other flavonoids are antioxidants found in fresh fruits and vegetables that have been shown to play an important preventative role in cardiovascular diseases and aging.^{1–3} A number of enzymes have been found which decompose flavonoids by reaction with dioxygen, Scheme 1.^{4,5} The same reaction occurs non-enzymatically, though typically at higher pH.⁴

In a series of mechanistic studies, the Speier group proposed that in organic solvents the non-enzymatic reaction proceeds by a way of a rate-limiting single electron transfer (SET) between the deprotonated flavonol and oxygen, but in protic solvents a second slower bimolecular reaction competes.⁵ Metal complex models for the enzymatic reactions have also been examined by Speier⁶ and others.^{7,8} In both the free or metal-bound non-enzymatic oxygenations, the reactions are typically observed at temperatures above 70 °C, with reported activation free energies of greater than 24.1 kcal/mol.^{5a,6f}

Nitroxyl, HNO, is the reduced and protonated congener of NO, which is isoelectronic with singlet O_2 . HNO displays biological effects distinct from that of NO, for example, as an enzyme inhibitor⁹ and ionotropic agent that may be used in the treatment of heart failure.¹⁰ Recently, we reported the unprecedented substitution of HNO for dioxygen in the activity of Mn-substituted Quercetin Dioxygenase, Mn-QDO, resulting in the incorporation of both heteroatoms of HNO regioselectively into the product, Scheme 2.⁹ In these reactions, HNO is generated in situ from a precursor, and in the presence of enzyme and substrate, and like dioxygenation, cleaves the central O-heterocyclic ring to release CO. The reaction likely proceeds through an analogous depsidic product **2**, which



Scheme 1.



Scheme 2.

decomposes to give the observed 2,4,6-trihydroxybenzoic acid and 3,4-dihydroxybenzonitrile products. Importantly, like dioxygenation, a non-enzymatic nitroxygenation of quercetin with HNO proceeds at high pH yielding the same regioselective products, again suggesting the deprotonated quercetin anion is the dominant reactant.

The coupling of HNO to an enolic carbon center is similar to the so-called nitroso aldol reactions, NA, in which nitroso compounds couple with activated ketones and aldehydes yielding both O- and N-bound adducts, Scheme 3.¹¹ The early examples of these aldol condensations utilized nitrosobenzene and strongly activated enolates or silyl enol ethers, typically yielding N-bound

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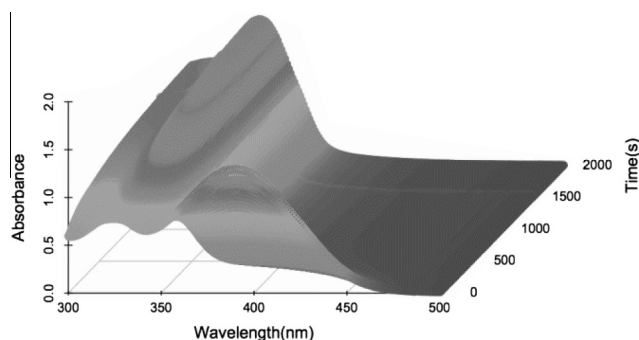
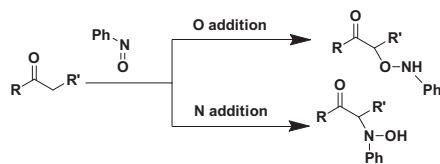


Figure 1. Absorbance spectra obtained over the course of reaction of quercetin (0.04 mM) with HNO donor Angeli's salt (1.00 mM) in pH 8.0 sodium phosphate buffer at 298 K.

Table 1
Temperature dependence of AS decomposition

T (K)	k_1 (s ⁻¹) ^a	
	HNO	DNO
288	5.0×10^{-4}	4.0×10^{-4}
293	7.0×10^{-4}	6.0×10^{-4}
298	1.8×10^{-3}	1.5×10^{-3}
303	2.6×10^{-3}	2.3×10^{-3}
308	3.4×10^{-3}	2.8×10^{-3}

^a Experimental variance within 5%.

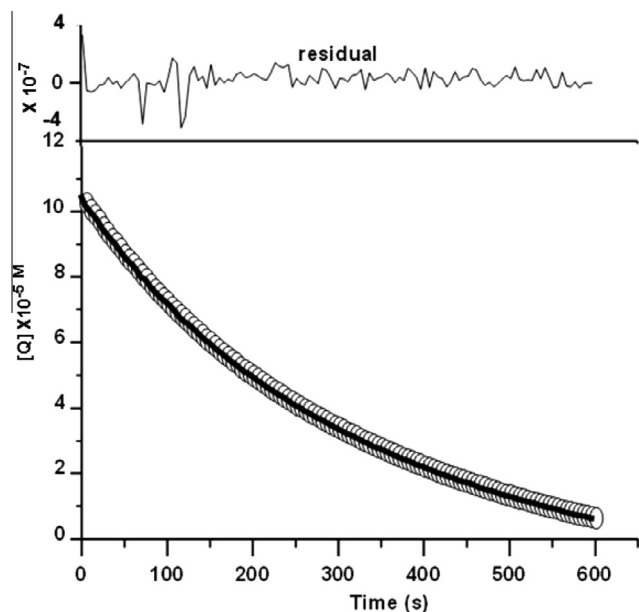


Figure 2. Overlay of modeled (dot) and experimentally-derived (dash) concentrations for a typical reaction, with residual plot above.

Table 2
Temperature dependence of k_3

T (K)	k_3 (M ⁻¹ s ⁻¹) ^a × 10 ⁴	
	HNO	DNO
288	0.782 (±0.15)	0.418 (±0.13)
293	1.10 (±0.05)	0.573 (±0.08)
298	1.34 (±0.09)	0.852 (±0.07)
303	1.85 (±0.07)	1.32 (±0.05)
308	2.49 (±0.12)	2.06 (±0.09)

^a Experimental variance is within 5%, average of three trials (error in parenthesis).

Table 3
Determined activation parameters at 20 °C for Eq. 3

	$\Delta H^\ddagger$ (kcal/mol)	$\Delta S^\ddagger$ (cal/mol K) ^a	$-T\Delta S^\ddagger$ (kcal/mol)	$\Delta G^\ddagger$ (kcal/mol) ^b
HNO	9.38 (±0.04)	-8.27 (±0.03)	2.42	11.80
DNO	13.61 (±0.02)	5.07 (±0.04)	-1.48	12.13

^a From intercepts of the Eyring plots.

^b From the equation $\Delta G^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger$.

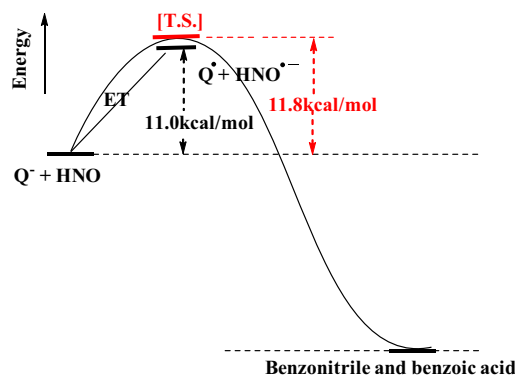
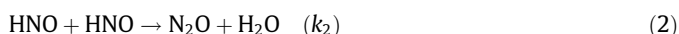
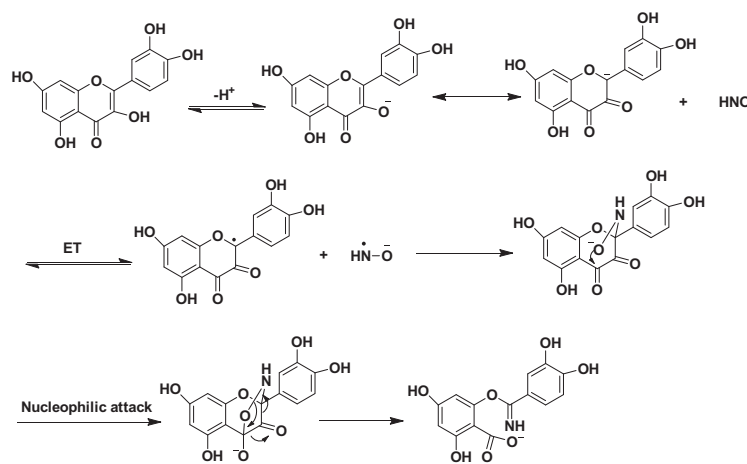


Figure 3. Reaction coordinate diagram for Eq. 3 showing determined activation energy in red, and calculated value for initial outer-sphere electron transfer mechanism.

hydroxyamino products.^{12–14} More recent work by Yamamoto and coworkers have shown a much wider scope of NA reactivity,¹⁵ with Lewis-acid catalysis yielding O-bound aminooxy adducts.^{16,17} These reactions are proposed to occur through bimolecular nucleophilic attack, rather than outersphere SET.

In this Letter, we investigate the kinetics and thermodynamics of the non-enzymatic reaction to address questions regarding the mechanism of nitroxygenation. Reactions between quercetin and HNO were monitored by the decay of the quercetin absorption band with λ_{max} of 400 nm, as shown in Figure 1. The majority of experiments were run at pH 8.0 in phosphate buffer, conditions at which quercetin ($pK_a = 7.1$)¹⁸ is almost 90% deprotonated and compatible with the use of Angeli's Salt (AS) as a stable source of HNO. Under analogous conditions, no side reaction of quercetin with the byproduct NO_2^- , was seen on the timescale of the measured reactivity.





Scheme 4.

The reaction sequence in Eqs. 1–3 was used to model the kinetic data. These reactions are complicated by the slow release of HNO from AS, and its competitive dimerization forming N_2O .¹⁹ The temperature dependence under our conditions for the first-order rate constant k_1 for Eq. 1 was obtained following the loss of absorbance of Angeli's salt at 250 nm, in both H_2O and D_2O solutions, Table 1. A literature value for the bimolecular rate of Eq. 2, $k_2 = 8 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, was assumed unchanged under these conditions.^{9,19}

The temperature dependence of the second-order rate constant of Eq. 3, k_3 , was obtained by converting absorbance data to concentration versus time and then fitting the data to the reaction sequence using REACT for Windows, Version 1.2, Figure 2.²⁰ Table 2 gives the determined rate constants for analogous reactions of HNO and DNO, the latter being the assumed reactant in D_2O solution.

Using the data in Table 2, the activation enthalpy ($\Delta H^\ddagger$) and activation entropy ($\Delta S^\ddagger$) for the reaction were derived from Eyring plots of $\ln(k_2/T)$ versus the reciprocal of the absolute temperature ($1/T$), respectively, and are given in Table 3 for reactions in both H_2O and D_2O . Analysis of the variable temperature kinetic data on this reaction obtains an activation free energy $\Delta G^\ddagger$ of 11.8 kcal/mol, shown in Table 3. The kinetic isotope effect, KIE, derived from rate constants obtained in H_2O and D_2O solutions is 1.92 at 293 K. This value suggests, as well as the relatively small entropic energies of activation, suggest that no bonds are made or broken in the rate-determining step.



For comparison, the bimolecular rate constant of dioxygenation, k_4 , at 293 K is $0.46 \text{ M}^{-1} \text{ s}^{-1}$, ca. 10^4 slower than that of nitroxylation. Likewise, the reported enzymatic dioxygenation reaction of quercetin by Mn-QDO was ca. 10^4 slower than the analogous nitroxylation.^{9,4a} Thus nitroxylation appears to be fundamentally and significantly more facile than dioxygenation in these reactions.

$$\Delta G^\circ = F[E^\circ(\text{QH}^{+/0}) - E^\circ(\text{HNO}^{0/-})] \quad (5)$$

Further insight is obtained by application of the Nernst relationship, Eq. 5, utilizing the reported potentials versus NHE for quercetin oxidation (0.26 V) and HNO reduction (-0.22 V),^{21–23} which obtains the theoretical energy for single electron transfer (SET) of 11.0 kcal/mol. This value is quite close to that of the determined $\Delta G^\ddagger$ for the non-enzymatic nitroxylation, and thus is consistent with an initial SET as the rate-determining step, as illustrated in Fig. 3. One product of SET would be $\text{HNO}^\bullet$, the aminosyl radical

anion, at an estimated reduction potential of 0.52 V NHE,²⁴ as compared to that of superoxide at -0.33 V from O_2 , which would be required for dioxygenation by SET.²⁵ Thus the accelerated rate of nitroxylation versus oxygenation can be attributed to the difference in driving force in the SET rate-determining step.

These results are consistent with the proposed mechanism shown in Scheme 4. In this sequence, quercetin is first deprotonated, generating an anion with substantial electron density located on C2 of the central ring. The rate-limiting electron transfer between quercetin anion and HNO produces a quercetin radical and the aminosyl radical anion, $\text{HNO}^\bullet$. Radical–radical coupling generates an N-oxyamino anion; this key intermediate may also be generated by nucleophilic attack of a C2-based carbanion on HNO, analogous to the nitroso aldol reactions. But the relatively small entropy of activation suggests no bond formation in the rate-determining step, thus supporting the outersphere SET mechanism.^{26,27} The incipient N-oxyamino anion then undergoes an intramolecular nucleophilic attack at the C4 carbonyl, releasing CO and forming the proposed depsidic intermediate which decomposes to the observed products.

Conclusion

The nitroxylation of quercetin with HNO is much more facile than the comparable dioxygenation, some 1000-fold faster at room temperature than an analogous dioxygenation at 70°C . Thermodynamic analysis yields an activation barrier very similar to that predicted for a rate-determining SET step; the difference in rates may be attributed to a large difference in SET driving force. The unique regioselectivity of nitroxylation may be of use in organic synthesis, as well as to provide mechanistic insight in comparison to analogous dioxygenation reactions.

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