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Mechanism of cysteine oxidation by peroxynitrite: An integrated experimental and theoretical study

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ABSTRACT

Since peroxynitrite was identified as a pathophysiological agent it has been implicated in a great variety of cellular processes. Particularly, peroxynitrite mediated oxidation of cellular thiol-containing compounds such as Cys residues, is a key event which has been extensively studied. Although great advances have been accomplished, the reaction is not completely understood at the atomic level. Aiming to shed light on this subject, we present an integrated kinetic and theoretical study of the oxidation of free Cys by peroxynitrite. We determined pH-independent thermodynamic activation parameters, namely those corresponding to the reaction between the reactive species: Cys thiolate and peroxynitrous acid. We found a pH-independent activation energy of 8.2 ± 0.6 kcal/mol. Simulations were performed using state of the art hybrid quantum–classical (QM–MM) molecular dynamics simulations. Our results are consistent with a S_N2 mechanism, with Cys sulfenic acid and nitrite anion as products. The activation barrier is mostly due to the alignment of sulfur's thiolate atom with the oxygen atoms of the peroxide, along with the concomitant charge reorganization and important changes in the solvation profile. This work provides an atomic detailed description of the reaction mechanism and a framework to understand the environment effects on peroxynitrite reactivity with protein thiols.

Introduction

Oxidation of free Cys,[1] GSH or Cys residues in proteins, is a key event implicated in a great variety of cellular processes such as antioxidant responses, signal transduction, regulation of the activity of enzymes, protein channels and/or transcription factors [1–6]. Peroxynitrite (as the sum of peroxynitrite anion (−1) and peroxinitrous acid)[2] is formed in the cell by the fast reaction between superoxide anion ($O_2^{\cdot-}$) and nitric oxide ($^{\cdot}NO$) radicals, with a second order rate constant of about 10^9–10^{10} M^{-1} s^{-1} [7,8]. ONOOH has a *pKa* between 6.5–6.8 [7–9], and decays producing hydroxyl ($^{\cdot}OH$) and nitrogen dioxide ($^{\cdot}NO2$) radicals in ~30% yields ($k = 0.9$ s^{-1}, pH 7.4, 37 °C) [5,8]. Both ONOOH and $ONOO^-$ are strong oxidants which react with different cell molecular components and since the first proposal as a pathophysiological agent [11–14], these species have been implicated in a numerous biologically relevant processes associated with protein function modification and cellular signaling among others (for comprehensive reviews see Ref. [15–16]). Particularly, the two-electron oxidation of free Cys by peroxynitrite has been studied from a kinetic approach [14,17,18]. The process is actually carried out by the thiolate form of Cys ($CysS^-$) and ONOOH [9,18], through the reaction:

$$CysS^- + ONOOH \rightarrow RSOH + NO_2^- \quad (1)$$

The second order rate constant has been reported as 5–6 × 10^3 - M^{-1} s^{-1} (37 °C), with an activation energy of about 9.7 kcal/mol (pH 7.5) [14,17]. The reaction has been proved to be strongly pH-dependent [14].

Although the reactivity of low molecular weight thiols and some protein Cys residues like human serum albumin can be related with thiol acidity constants, some proteins like peroxiredoxins or glyceraldehydes 3-phosphate dehydrogenase show a much higher reactivity than the expected just from inspecting thiol's *pKa*, [19–22] indicating that besides this factor, the environment surrounding thiol groups is critical to comprehend this phenomenon and it is important to get a molecular viewpoint of this reaction.

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[1] *Abbreviations used:* Cys, L-cysteine; $CysS^-$, cysteinate; CysSOH, cysteine sulfenic acid; GSH, glutathione; DFT, density functional theory; QM, quantum mechanics; MM, molecular mechanics; MD, molecular dynamics; dzvp, double zeta valence with polarization; IRC, intrinsic reaction coordinate.

[2] The IUPAC recommended names for peroxynitrite anion and peroxynitrous acid are, oxoperoxonitrate (1-) and hydrogen oxoperoxonitrate, respectively.

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In order to contribute to this understanding, we present here an integrated kinetic and theoretical approach of the oxidation of cysteine presented in Eq. (1). pH-independent thermodynamic activation parameters were determined from kinetics experiments. The reaction mechanism and system properties were evaluated on the basis of hybrid QM–MM MD simulations combined with an umbrella sampling scheme, which allow us to achieve free energies and the evolution of the electronic properties along the reaction coordinate, within a realistic representation of the aqueous environment [23]. This work represents the first theoretical study of this important reaction. Our results underline the pH dependency of the process and the solvent significance, assisting in the orientation of ONOOH and allowing the charge reorganization to take place. The data presented herein set the basis for further integrated studies on the mechanism of thiol oxidation in different protein environments.

Materials and methods

Chemicals

L-Cysteine, diethylenetriaminepentaacetic acid (dtpa), 5,5′-dithiobis(2-nitrobenzoate) (DTNB), and sodium phosphate salts were purchased from Sigma–Aldrich. Peroxynitrite was synthesized from H_2O_2 and nitrous acid as described previously [11,14]. Stock solutions of peroxynitrite were treated with granular manganese dioxide to eliminate remaining H_2O_2. Nitrite contamination was typically < 30% of peroxynitrite concentration [24].

Kinetics experiments

All experiments were performed in 100 mM sodium phosphate buffer containing 0.1 mM dtpa. Differential mixing of mono- and di-basic phosphate buffers were performed in order to achieve different pHs. Ionic strength was kept constant (0.15 mM) with the addition of NaCl.

Peroxynitrite concentration was determined at alkaline pH at 302 nm (ε_{302} = 1670 $M^{-1}cm^{-1}$) and was equal to 0.1 mM in the mix. Thiol content of Cys solutions was measured by Ellman's assay (ε_{412} = 14150 $M^{-1}cm^{-1}$) [25] and was varied between 6.0 and 13.0 mM in the final mix.

Kinetics of peroxynitrite decomposition was followed by absorbance spectroscopy (λ = 302 nm) in an Applied Photophysics SX-20 stopped-flow spectrofluorimeter (mixing time of $\leqslant$ 1.2 ms). Outlets pH measurements were always done at bath temperature (namely 10, 25, 37 and 50 °C). Each observed rate constant, which correspond to pH-dependent first order rate constants (k_{1pHdep}) of peroxynitrite decay, was determined by fitting at least 5 data sets to single exponential functions. Firstly, ONOOH auto-decay pH-independent rate constants were determined for each temperature, fitting k_{1pHdep} constants versus pH:

$$k_{1pHdep} = k_{1pHindep}\left(\frac{10^{-pH}}{10^{-pKa_{ONOOH}} + 10^{-pH}}\right) \quad (2)$$

Then, pH-dependent second order rate constants (k_{2pHdep}) for the reaction between cysteine and peroxynitrite were determined, following peroxynitrite decay in the presence of excess Cys under different conditions of pHs and temperatures. Experimental data were fitted to single exponentials, from which k_{obs} values were obtained.

Since in this case $k_{obs} = k_{1pHdep} + k_{2pHdep}$ x [Cys], k_{2pHdep} were determined by subtracting the corresponding auto-decay rate at exactly the same pH and dividing by Cys concentration (two different Cys concentrations were usually used at each pH and temperature, with identical results). This method was preferred over rate constant determinations at multiple Cys concentrations at a single pH for each pH and temperature, to rigorously control pH values when using acidic stock solutions of Cys and to avoid Cys stock oxidation during the experiment that could occur if Cys stock solutions were neutralized. For selected pHs and temperatures, both methodologies were used yielding the same results.

As a result, pH-independent second order rate constants were calculated from the fitting of the plot of k_{2pHdep}versus pH:

$$k_{2pHdep} = k_{2pHindep}\left(\frac{10^{-pKa_{Cys}}}{10^{-pKa_{Cys}} + 10^{-pH}}\right)\left(\frac{10^{-pH}}{10^{-pKa_{ONOOH}} + 10^{-pH}}\right) \quad (3)$$

Classical Arrhenius and Eyring's analysis were then performed over the data obtained as explained above.

Computer simulations

Initial survey of the system

In order to obtain information about the energy surface and the mechanism of the reaction under investigation, and to carry out a methodology evaluation, we performed several electronic structure calculations using *Gaussian 03* [26]. The structures of reactants ($CysS^-$ and ONOOH) and reactant complex (RC) ($CysS^-$/ONOOH), products complex (PC) (CysSOH/NO_2^-) and transition state (TS) were optimized both in vacuo and in the presence up to 4 water molecules at different levels of theory: *HF*, *PBE*, *B3LYP*, *MP2*, employing a double-zeta plus polarization (*dzvp*) Gaussian basis set [27]. Frequency calculations were performed in all cases. Aiming to investigate if one or more water molecules could be involved in the reaction mechanism, we also performed IRC calculations at the *PBE/dzvp* level of theory including one and four water molecules in the QM system.

ONOOH *B3LYP* calculation was used to get classical parameters of this moiety [28], necessaries to perform the classical MD simulations which are required to equilibrate the systems, as described below.

QM–MM molecular dynamics simulations

The actual QM–MM simulations were carried out using the code and parameters described in Ref. [23] (for details on the QM–MM scheme see Refs. [29–30]). The system consisted in the quantum solute ($CysS^-$ + ONOOH) embedded in a box containing 3247 classical TIP4P water molecules. For the QM region, computations were performed at the generalized gradient approximation (GGA) level, using the *PBE* combination of exchange and correlation functionals, with a *dzvp* basis set for the expansion of the one-electron orbitals [27]. All the QM–MM MD simulations were run for at least 5 ps and employed the Verlet algorithm to integrate Newton's equations with a time step of 0.2 fs. Initial configurations were generated from preliminary 100 ps classical equilibration runs in which the solute was treated classically as a rigid moiety, followed by a QM–MM MD where the solute was treated at the *AM1* semi-empirical level, as implemented in Amber [31].

To explore reaction's free energy and mechanism, we employed an umbrella sampling scheme, choosing as reaction coordinate the difference between the O_1–O_2 and the S–O_1 distances (see Fig. 2), which was sampled from −1.8 to 1.0 Å, divided in 29 simulations windows.

All dynamics visualizations and molecular drawings, were performed with VMD 1.8.6.32.

Results and discussion

pH-independent reaction parameters

The kinetics of the oxidation mediated by peroxynitrite of low molecular weight thiols like Cys and GSH [14,17] or Cys residues