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Evidence for Protein Radical-Mediated Nuclear Tunneling in Fatty Acid α-Oxygenase

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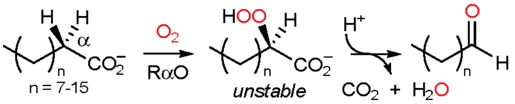
The α oxidation of fatty acids is associated with essential metabolic, protective, and cell signaling functions in higher organisms.[1,2] Here we provide evidence for an α oxidation mechanism involving a protein-derived radical. The reaction of interest is the first step in eq 1 where 2-*R*-hydroperoxide compounds are formed upon insertion of O_2 into fatty acid C_α−H bonds. In heme-containing dioxygenases,[1] such as rice α-oxygenase (RαO), the catalytic oxidant is proposed to be a tyrosyl radical.[1c] This idea derives from (i) the homology of RαO to cyclooxygenases (COX-1 and COX-2) believed to use a Tyr• to oxidize arachidonic acid to prostaglandin H^3 and (ii) the loss of activity upon mutation of the conserved Tyr379 to Phe.[1c,4−6]



(1)

We have found that treatment of RαO with H_2O_2 enhances the enzyme's activity and results in formation of a persistent organic radical.[6] Experiments under anaerobic conditions suggest that the Tyr379• is thermodynamically capable of H• abstraction from the fatty acid C_α−H. Large, weakly temperature dependent deuterium kinetic isotope effects (KIEs, ${}^{D}k_{cat}$ and ${}^{D}k_{cat}/K_M$) are observed, consistent with nuclear tunneling. The description of such quantum effects poses an important challenge for current theories of proton-coupled electron transfer.[7,8]

Though a crystal structure is not yet available,[9] we have constructed a model of RαO using a COX-1 mutant with ∼15% homology (Figure 1).[10] Overlaying the RαO model structure with the monomeric subunit of wild-type (wt) COX-1 reveals similarly positioned His ligands to the Fe^{III} protoporphyrin IX (Fe^{III}Por) and virtually identical positions for the proposed catalytic Tyr residues (∼6 Å from the porphyrin edge). In spite of overlapping Arg residues, the fatty acid is expected to bind differently in RαO with C_α−H pointing toward Tyr379 possibly due to an interaction between the carboxylate and His311.

Exposure of Fe^{III}-RαO to H_2O_2 results in oxidation of Tyr379 but not exogenous reductants;[1c] this is a marked contrast to the peroxidase activity of COX.[3] Reacting RαO (50 μM) with H_2O_2 under N_2 results in an electron paramagnetic resonance (EPR) signal that persists for ≥1 h (Figure 2a). The spectral features[6,12] at ambient and low temperatures are consistent with a Tyr• in a yield of ∼25% spin/heme after 20 min. The signal is diminished upon adding substrates such as decanoic or palmitic acids during preparation but not the inhibitor 5-(tetradecyloxy)-2-furoic acid (TOFA), which does not possess C_α−H bonds (Figure 2b).[6] The putative substrate-derived radical is not detectable.

The Tyr379Phe mutant does not form a stable protein radical upon treatment with H_2O_2 (Figure 2c). The reactions of the wt and mutant RαO also differ spectrophotometrically (Figure 2d,e). Under conditions analogous to those of the EPR experiment, a red shift and slight bleach of the Soret band occur rapidly in wt-RαO.[13] In contrast, Tyr379Phe RαO forms an intermediate resembling Fe^{IV}(O)Por•+ which is reduced to Fe^{IV}(O)Por. Three Trp residues in the active site may provide the reducing equivalent(s).

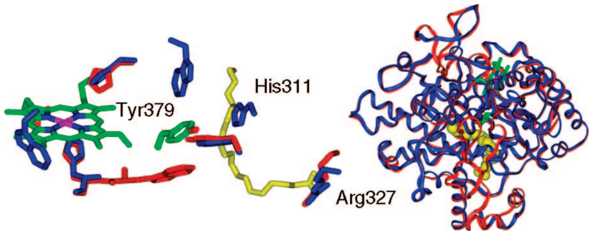


Figure 1. Model of the active site of RαO (green, blue) compared to COX-1 (red) with bound arachidonate (yellow) (PBD: 1DIY).[11]

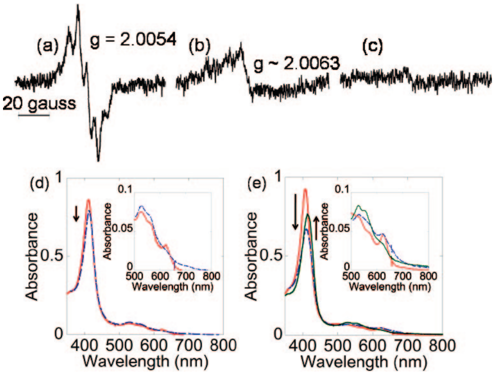


Figure 2. X-band EPR spectra (22 °C) after 20 min (center = 3450 G): (a) wt-RαO (50 μM) + 10 equiv of H_2O_2. (b) Sample (a) + 40 equiv of decanoate. (c) Tyr379Phe RαO (50 μM) + 10 equiv of H_2O_2. UV−vis absorption spectra: (d) wt-RαO (7 μM) + 10 equiv of H_2O_2 (initial = red, final = blue). (e) Tyr379Phe RαO (7.5 μM) + 10 equiv of H_2O_2, (initial = red, intermediate = blue, final = green).

Steady-state kinetics using an O_2 electrode reveals that palmitate and decanoate are fast and slow substrates, respectively, with K_M values that vary by $>10^2$. Full enzyme activity was ensured by preincubating RαO with H_2O_2 (10−50 equiv) prior to the initial rate measurements. Experiments with peroxidases and reducing cosubstrates indicate that peroxide impurities present in the reaction mixtures are required for enzyme activity.[3,6]

The kinetics of palmitate oxidation is only slightly affected by changes in pH: at pH 10, $k_{cat} = 18 \pm 1.5$ s^{-1}, k_{cat}/K_M(palmitate) = $(3.5 \pm 1.0) \times 10^6$ M^{-1} s^{-1} and $k_{cat}/K_M(O_2) = (4.0 \pm 0.5) \times 10^5$ M^{-1} s^{-1}; at pH 7.2, $k_{cat} = 9.0 \pm 0.5$ s^{-1}, k_{cat}/K_M(palmitate) = $(2.2 \pm 0.4) \times 10^6$ M^{-1} s^{-1} and $k_{cat}/K_M(O_2) = (3.0 \pm 0.5) \times 10^5$ M^{-1} s^{-1}. Errors are reported throughout as $\pm 1\sigma$. Experiments were mostly conducted at the higher pH where enzyme activity and substrate solubility are enhanced, while kinetic complexity appears to be minimized (see below). Under the experimental conditions, the 2-*R*-

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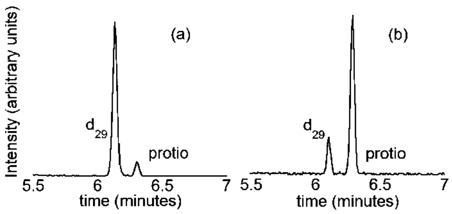

Figure 3. Determination of ${}^{D}k_{cat}/K_M$ by GC/MS analysis. The resolution of d_{29} and h_{29} pentadecanal at (a) 100% and (b) ~1% product formation.

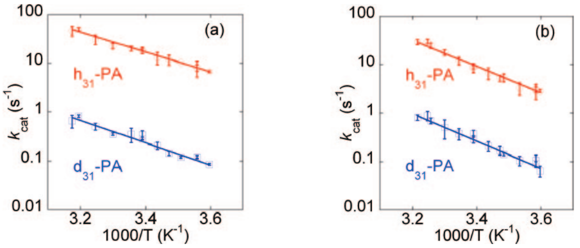

Figure 4. Temperature dependence of k_{cat} for h_{31}- and d_{31}-palmitate at pH 10 (a) and pH 7.2 (b). Errors are shown for visibility as $\pm 2\sigma$.

hydroperoxide products are unstable[1c] losing CO_2 to afford C_{N-1} aldehydes which were identified and quantified using gas chromatography/mass spectrometry (GC/MS).

Palmitate oxidation is characterized by a large competitive KIE at pH 10. Experiments were performed by analyzing the product ratios from solutions initially containing 5:1 or 10:1 mixtures of h_{31}:d_{31}-palmitate (Figure 3).[14] Five independent determinations at <5% reaction conversion indicated ${}^{D}k_{cat}/K_M$ (22 °C) = 53 ± 5.

Large KIEs were also determined uncompetitively at pH 10 with saturating ($\geq 10K_M$) concentrations of O_2 and palmitate (>98% isotopic purity).[6] Identical k_{cat} values were observed in H_2O and D_2O indicating the absence of isotope exchange into the α-position. ${}^{D}k_{cat}$(palmitate) = 54 ± 7 (22 °C) was determined using α,α-d_2-palmitate or d_{31}-palmitate. None of the experiments showed a kinetic burst due to protio substrate contamination or an induction phase due to insufficient peroxide initiator.[6] The similar KIEs, ${}^{D}k_{cat}$ and ${}^{D}k_{cat}/K_M$, imply that C_α−H cleavage is the first irreversible as well as the turnover-controlling step at pH 10. At pH 7.2 and 22 °C, ${}^{D}k_{cat}$ is diminished to 31 ± 5. This is presumably the result of a downstream unimolecular step that contributes to k_{cat}, reducing the KIE from the intrinsic value.

The temperature dependence of the intrinsic KIE reveals the quantum mechanical nature of C−H oxidation as discussed by Klinman et al.[15] Arrhenius plots reveal $E_a(H) = 9.3 \pm 0.2$ kcal mol^{-1}, $A(H) = (1.3 \pm 0.4) \times 10^8$ s^{-1}, $E_a(D) = 10.4 \pm 0.2$ kcal mol^{-1} and $A(D) = (1.3 \pm 0.3) \times 10^7$ s^{-1} at pH 10; $E_a(H) = 12.6 \pm 0.3$ kcal mol^{-1}, $A(H) = (2.1 \pm 0.9) \times 10^{10}$ s^{-1}, $E_a(D) = 12.9 \pm 0.3$ kcal mol^{-1} and $A(D) = (1.1 \pm 0.6) \times 10^9$ s^{-1} at pH 7.2 (Figure 4). The intrinsic KIE is more fully expressed at pH 10 where $E_a(D) - E_a(H) = 1.1 \pm 0.3$ kcal mol^{-1} and $A(H)/A(D) = 10 \pm 4$. The $A(H)/A(D)$ deviates from 1 within the $\pm 1\sigma$ error weighted limits, as seen in some other systems.[6,15c,d]

This study has demonstrated large deuterium KIEs upon C−H oxidation by RαO and supported the intermediacy of the Tyr379• in catalysis. Though Fe^{IV}(O)Por•+ and Fe^{IV}(O)Por were observed, a persistent radical was absent in the Tyr379Phe mutant for which no activity could be detected in solutions containing up to 5 μM protein. Future efforts will concentrate on the kinetic mechanism. At this stage, we cannot rigorously exclude the possibility that the Tyr379• is a side product or the intermediacy of another catalytic oxidant, possibly an unstable peroxyl radical. A reversible reaction with O_2 to form an amino acid peroxyl radical that is regenerated with each enzyme turnover would still be consistent with the stoichiometry of eq 1. Consideration of such a species is warranted in view of the thermodynamics; the H• affinity of a Tyr• is expected to be less than the inverse bond dissociation energy of the fatty acid C_α−H (~90 kcal mol^{-1}) making initial H• abstraction endothermic.[16]

Though increasingly observed in enzymes that oxidize substrate C−H bonds,[15] the large KIEs in RαO are somewhat surprising. The analogous reaction in the homologous COX-1 is characterized by a small tritium KIE of ~4.[3] Kinetically reversible H• abstraction by the Tyr• has been proposed in COX-1,[17] a reaction that involves oxidation of a weak, bis-allylic C−H with a dissociation energy of ~80 kcal mol^{-1}. The irreversible reaction in RαO suggests that the oxidant's thermodynamic affinity for H• may be significantly altered relative to COX.

RαO, thus, presents a novel example where a protein-derived radical may effect homolysis of a robust C−H bond by nuclear tunneling.[8] Although a close distance between H• donor and acceptor is anticipated for radical reactions,[7c] the KIEs from 40 to 60 (5−40 °C) reveal nonadiabatic behavior due to vibrational overlap below the activation barrier. In view of these unique results, it will be interesting to determine how current theories of proton-coupled electron transfer are able to account for the isotopic activation parameters seen in RαO.[7]

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Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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