Pulsed Dipolar Spectroscopy Reveals That Tyrosyl Radicals Are Generated in Both Monomers of the Cyclooxygenase-2 Dimer

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Supporting Information

ABSTRACT: Cyclooxygenases (COXs) are heme-containing sequence homodimers that utilize tyrosyl radicalbased catalysis to oxygenate substrates. Tyrosyl radicals are formed from a single turnover of substrate in the peroxidase active site generating an oxy-ferryl porphyrin cation radical intermediate that subsequently gives rise to a Tyr-385 radical in the cyclooxygenase active site and a Tyr-504 radical nearby. We have utilized double-quantum coherence (DQC) spectroscopy to determine the distance distributions between Tyr-385 and Tyr-504 radicals in COX-2. The distances obtained with DQC confirm that Tyr-385 and Tyr-504 radicals were generated in each monomer and accurately match the distances measured in COX-2 crystal structures.

he cyclooxygenases (COX-1 and COX-2) are the primary targets of nonsteroidal anti-inflammatory drugs (NSAIDs) and COX-2 selective inhibitors (coxibs). COX enzymes are heme-containing sequence homodimers. Each monomer contains two active sites: a cyclooxygenase active site that catalyzes the bis-dioxygenation of arachidonic acid (AA) to form the intermediate prostaglandin G_2 (PGG₂), and a peroxidase active site that reduces the 15-hydroperoxide group of PGG_2 to form prostaglandin H_2 (PGH_2). COX requires a preliminary catalytic turnover at the peroxidase active site to generate an oxy-ferryl porphyrin cation radical (Figure S1). The radical is subsequently transferred to Tyr-385 located in the cyclooxygenase active site, which abstracts the 13-pro-S hydrogen from AA to initiate cyclooxygenase catalysis (Figure S1).¹ PGH₂ is metabolized by downstream tissue specific synthases to form potent lipid signaling molecules involved in regulating physiological homeostasis. These molecules also play intricate roles in pathological states such as inflammation and cancer.1,2

Electron spin resonance (ESR) spectroscopic studies have shown that a radical is also formed on Tyr-504 during cyclooxygenase catalysis.^{3,4} Reaction of Y385F COX-2 with a peroxide substrate generates a radical that is localized solely on Tyr-504.⁴ Tyr-504 is located near the proximal heme ligand just outside of the cyclooxygenase active site (Figure S2) and is not directly involved in catalysis, as Y504F COX-2 retains wild-type levels of activity.⁴ While the role of the Tyr-504 radical is not clear, it has been proposed by Rogge and colleagues that an equilibrium exists between the radicals on Tyr-385 and Tyr-504 (Figure S2), with Tyr-504 serving as a "radical reservoir" that replenishes the catalytic Tyr-385 radical after depletion by a reductant.⁴ Importantly, previous continuous wave ESR studies have not provided information about the spatial location of tyrosyl radicals with respect to one another in the COX dimer. Mutational studies using a heterodimer of COX-2 in which one monomer lacked peroxidase activity and the partner monomer lacked cyclooxygenase activity resulted in a cyclooxygenase deficient enzyme, demonstrating that electron transfer across the dimer interface does not occur.⁵ Thus, for the radical reservoir hypothesis to hold true, Tyr-385 and Tyr-504 radicals must be generated within the same monomer, which has yet to be demonstrated experimentally.

A new paradigm has emerged with respect to COX catalysis and regulation. In this model, COX functions as a conformational heterodimer with only one monomer active at a given time.⁶ Dietary nonsubstrate fatty acids and certain NSAIDs bind to one monomer, the "allosteric" monomer (E_{allo}) , to modulate substrate oxygenation in the partner "catalytic" monomer (E_{cat}) .^{6,7} The mechanism governing intermonomer communication is unknown, and it remains unclear if tyrosyl radicals play a role in allosteric regulation. A potential scenario in which a catalytic Tyr-385 radical is generated in only one monomer has not been ruled out (Figure S2).^{6,8} This scenario is particularly intriguing, as it would provide an explanation for the half-of-sites reactivity observed with COX-2. A complete understanding of COX half-of-sites reactivity and allostery is necessary, as current investigations are trending toward the design of next-generation NSAIDs that inhibit COX in a substrate selective and allosteric manner.

Pulsed ESR techniques such as double electron–electron resonance (DEER) and double-quantum coherence (DQC) are powerful methods for extracting distance information between paramagnetic species in proteins.¹⁰ While these techniques are

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often utilized in combination with nitroxide spin-labels that are introduced into proteins via site-directed spin labeling, their utility has been demonstrated with additional paramagnetic cofactors. Four-pulse DEER has previously been utilized to determine inter-tyrosyl radical distances on the order of 33 Å in ribonucleotide reductase¹¹ and 52 Å in ψ factor-producing oxygenase A.¹² In this investigation, we utilized DQC to determine the spatial distribution of tyrosyl radicals in the COX-2 dimer.

Wild-type, Y385F, and Y504F human COX-2 constructs were expressed and purified with 0.1% Tween 20 (v/v) utilized as the solubilization and purification detergent (Supplemental Methods).¹³ The constructs, at concentrations of 150 μ M monomer, were reconstituted with a 1.5-fold molar excess of heme, followed by the addition of glycerol to a final concentration of 20% (v/v). Each construct was then reacted on ice with a 10-fold molar excess of the alkyl peroxide 15hydroperoxyeicosatetraenoic acid to generate tyrosyl radicals before being flash-frozen in liquid nitrogen. DQC data were recorded at 17.3 GHz and 5 K on a home-built Ku-band pulse ESR spectrometer^{14,15} as described in the Supplemental Methods. Distance distributions were reconstructed from the time domain traces using the L-curve Tihkonov regularization method¹⁶ and refined with the maximum entropy method¹⁷ (see also Supplemental Methods). UV/vis titration of apo COX-2 with heme was performed to determine the stoichiometry of heme binding (Figure S3).

In the Y504F construct, tyrosyl radicals can be generated on only Tyr-385, giving rise to a single interspin distance across the dimer interface. The distance between Tyr-385 side chains in each monomer in crystal structures of murine (mu) COX-2 is 44.7 Å (Figure 1A).¹³ The distance distribution determined with DQC for the Y504F construct displays an average distance of ~45 Å, corresponding to the distance measured from the



Figure 1. DQC distance measurements between Tyr-385 and Tyr-504 in Y385F and Y504F COX-2. Schematic of the COX-2 dimer, with the locations of (A) Tyr-385 and (B) Tyr-504 depicted as colored spheres. Measured distances between pairs across the dimer interface are indicated and marked with a red dotted line. Time domain DQC data for (C) Y504F COX-2 and (D) Y385F COX-2. Distance distributions for (E) Y504F COX-2 and (F) Y385F COX-2.

crystal structure (Figure 1E). Similarly, radical formation occurs on only Tyr-504 in the Y385F construct. The distance between Tyr-504 side chains across the dimer interface is 58 Å in crystal structures of muCOX-2 (Figure 1B). The distance distribution obtained with DQC for the Y385F construct shows a prominent peak at ~55 Å, corresponding closely to the distance measured in the crystal structure (Figure 1F). Taken together, these results confirm that Tyr-385 and Tyr-504 radicals are generated in both monomers of the COX-2 dimer.

On the basis of the results obtained with the Y385F and Y504F constructs, we predicted that tyrosyl radicals would be generated on Tyr-385 and Tyr-504 in both monomers of the dimer in the wild-type enzyme. This scenario gives rise to several possible interspin distances across the dimer interface, as well as an intramonomer distance between Tyr-385 and Tyr-504 (Figure 2A). An initial DQC experiment was performed to determine the distribution of tyrosyl radicals across the dimer interface. The resulting distance distribution had a prominent peak at a distance of ~43.4 Å, corresponding to the Tyr-385. Tyr-385 intermonomer distance and a smaller peak at ~51 Å on its high slope, corresponding to the Tyr-385.



Figure 2. DQC distance measurements between Tyr-385 and Tyr-504 in wild-type COX-2. (A) Schematic of the COX-2 dimer, with Tyr-385 and Tyr-504 depicted as colored spheres. Estimated distances between pairs across the dimer interface as well as the single intramonomer distance are indicated. (B) Time domain DQC data for wild-type COX-2 recorded on a 3.5 μ s time scale. Long distances (>40 Å) from tyrosyl radicals located in opposite monomers dominate the record. The short distance component, originating from Tyr-385 and Tyr-504 located in the same monomer, appears as a single point spike in the beginning of the record and is shown on an extended time scale in the inset for the sake of clarity. (C) Reconstructed distance distributions for the data in panel B. The distance distribution from 35 to 70 Å was fit to three Gaussian functions with the means indicated by arrows. The short distance component does not appear in the distribution (Supporting Information). (D) DQC data for the short distance component in panel B were recorded on a short time scale of 0.2 μ s and plotted after removing the small linear background (Supporting Information). (E) Distance distribution for intramonomer data of panel D.

intermonomer distance. No peak that corresponds to the Tyr-504·Tyr-504 intermonomer distance was resolved; however, the distribution does contain a contribution in this range. A fit to the distribution from 35 to 70 Å using three Gaussian functions was made to estimate contributions from the three intermonomer components (Supplementary Methods). The fit depicted in Figure 2C represents the experiment quite well and accurately reproduces the time domain DQC signal (Figure S6). These measurements provide experimental evidence that Tyr-385 and Tyr-504 radicals are generated simultaneously in both monomers of COX-2.

The time domain signal in Figure 2B is formed by long distances across the dimer interface but also shows the presence of a fast evolving component at the beginning of the record (plotted on an expanded scale). Only a short intramonomer distance can cause this contribution to the signal. Distance reconstruction in Figure 2C was focused on resolving long distances, with the short distance component being discarded in this distance reconstruction (Figure S4). Accordingly, an independent DQC experiment was conducted using a 2 ns dwell time and a 200 ns data record (Supplemental Methods). The resulting distance distribution is a sharp narrow peak at a distance of 11.2 Å, matching the same measured intramonomer distance between Tyr-385 and Tyr-504 in the mu COX-2 crystal structure (Figure 2E). Resolution of this short distance component further demonstrates that both Tyr-385 and Tyr-504 radicals are generated within a single monomer of COX-2.

Collectively, the DQC experiments provide experimental confirmation that Tyr-385 and Tyr-504 radicals are generated in both monomers of the COX-2 dimer. The distances calculated from the DQC experiments utilizing enzyme in solution are consistent with distances observed when COX-2 is confined to a crystal lattice and sets the stage for future experiments utilizing heterodimers of COX-2.^{6,13} Both DQC and crystallographic characterizations of COX-2 necessitate the use of relatively high concentrations of enzyme and heme (>50 μ M). Given the low dissociation constant reported for highaffinity binding of heme to COX-2,^{6,8} the expectation is that both monomers of the sequence homodimer will be chelated with heme. As we observe tyrosyl radical formation in both monomers, heme must be bound to both subunits under the experimental conditions utilized for DQC. Measurements of cyclooxygenase catalysis using an oxygen electrode utilize 5 μ M heme in the reaction cuvette.^{6,13} We further investigated heme binding by titrating COX-2 with heme (Figure S3). The increase in soret peak absorbance was linear up to 5 μ M heme, indicating saturation of both monomers of COX-2 at low micromolar concentrations. The heme titration result is consistent with both the DQC measurements presented here and previous crystallographic analyses.

To the best of our knowledge, this is the first report of distance measurements between pairs of tyrosyl radicals using DQC, as well as an example of accurate measurements of very short distances by pulse ESR. In particular, the Tyr-504 Tyr-504 intermonomer distance of \sim 58 Å is at the higher end of distances determined in a membrane protein with pulsed ESR methods and to the best of our knowledge is the longest distance measured between a pair of protein-derived tyrosyl radicals. These experiments highlight the utility of DQC in extracting difficult to measure distance distributions from fast relaxing organic radical cofactors, and the ability of these distance measurements to resolve complex biological questions.

ASSOCIATED CONTENT

S Supporting Information

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Supplementary methods and figures related to protein production and DQC analyses (PDF)

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Notes

The authors declare no competing financial interest.

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