Structural insights into the half-of-sites reactivity in homodimeric and homotetrameric metalloenzymes
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Abstract
Half-of-sites reactivity in many homodimeric and homotetrameric metalloenzymes has been known for half a century, yet its benefit remains poorly understood. A recently reported cryo-electron microscopy structure has given some clues on the less optimized reactivity of *Escherichia coli* ribonucleotide reductase with an asymmetric association of α2β2 subunits during catalysis. Moreover, nonequivalence of enzyme active sites has been reported in many other enzymes, possibly as a means of regulation. They are often induced by substrate binding or caused by a critical component introduced from a neighboring subunit in response to substrate loadings, such as in prostaglandin endoperoxide H synthase, cytidine triphosphate synthase, glyoxalase, tryptophan dioxygenase, and several decarboxylases or dehydrogenases. Overall, half-of-sites reactivity is likely not an act of wasting resources but rather a method devised in nature to accommodate catalytic or regulatory needs.

Introduction
The regulation of enzymatic catalysis is controlled using various strategies, such as substrate binding, allosteric sites, cofactors, protein partners, post-translational modifications, and conformational changes from the inactive or partially active state to the fully functional state. Here, we highlight a less-considered regulatory mechanism regarding either equivalent active sites showing seemingly less optimized reactivity or inequivalent active sites in higher-order protein quaternary structures to govern reactivity. This was first described in the early 1970s and later labeled as “half-site reactivity” or “half-of-sites reactivity” [1]. Many enzymes have been described to utilize higher oligomer states, where the monomers have identical primary structures that exhibit substrate-to-product stoichiometry of half or less, indicating nonequivalence among present active sites. This phenomenon ostensibly decreases enzyme efficiency and questions the extravagancy of those enzymes and, if purposely evolved, what benefit it imposes upon those that exhibit the half-of-sites reactivity behavior. In the following sections, we discuss insights inspired by recent structural studies of several oxygen-activating enzymes and some non-oxygen-dependent enzymes that appear to purposefully utilize half-of-sites reactivity for the benefit of catalytic need or regulation, which has invoked new thoughts.

Ribonucleotide reductase
Ribonucleotide reductases (RNRs) have long been studied for their importance in DNA biosynthesis and repair. These enzymes catalyze the reduction of all four ribonucleotide building blocks to DNA building blocks by reducing the 2′-OH while maintaining relatively balanced product pools [2–4]. There are three defined classes of RNRs [5], with class I enzymes requiring oxygen to generate a neutral tyrosyl free-radical in the smaller homodimer β2 subunit of the enzyme [6–8]. The active RNR complex is an α2β2 complex [9], where the α2 subunit contains the binding sites for substrate and effectors, and the β2 subunit harbors the crucial tyrosyl radical needed for catalysis. This β2 subunit should harbor two expected tyrosyl radicals/β2, yet a substoichiometric ratio of radicals/β2 is observed. The β-tail forms a conformationally gated radical transfer pathway to shuttle the radical from the β2 subunit 35 Å in distance to the reaction site in the substrate/effector-bound homodimer α2 subunit in *Escherichia coli* RNR based on a docking model [10,11]. This forms a thyl radical which initiates catalysis by abstracting a hydrogen atom from the nucleotide substrate (Figure 1a) [3,12]. Until recently, much of the knowledge gained regarding class I RNR chemistry has been based on a symmetric docking model of the α2β2 tetramer structure (Figure 1b) [11], which was supported by low-resolution (23 Å) small-
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are not simultaneously loaded with substrate. There are distinct types of nucleotide substrates and binding sites. If each β subunit contains a Tyr, as anticipated for the \textit{in vivo} system, what is the mechanism to prevent the radical transfer from the β subunit to an empty α subunit? The two subunits have little affinity to each other without substrate bound [15]. However, the affinity significantly increases when the α2 subunit is bound with a substrate/effecter and can form a transient, catalytically active α2β2 complex [16]. The two dimeric subunits must also dissociate after each turnover to allow product escape and enable subsequent thiol-redox or glutaredoxin-mediated, nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reduction of the disulfide bond between Cys225 and Cys462 (Figure 1a) to restore them to the initial thiol form for the next turnover [17]. Due to its transiency, understanding the less optimal activity of the active α2β2 homotetramer has remained elusive for several decades.

The transient nature of the α2β2 association rendered its acquisition difficult through traditional protein crystallographic approaches. Recently, by strategic use of site-directed mutagenesis and elegant chemical biology application of unnatural 2,3,5-F3-tyrosine amino acid incorporation into the β2 subunit [18,19], the structure of the active form of RNR was successfully trapped by cryo-EM [17]. The 3.6 Å resolution cryo-EM structure elucidates how the proton-coupled electron transfer pathway is constructed, stabilized, and disassembled (Figure 1c). The radical from the β2 subunit is bound asymmetrically to the α2 subunit [22,23]. More strategic use of unnatural amino acid incorporation to overcome conformational gating and spectroscopically characterize significant conformational changes of critical radical transfer residues, such as Tyr356 and Tyr731, in the ordered versus disordered state is also open to exploration [21]. While this structure and recent studies support half-of-sites reactivity in RNR, this phenomenon requires more thought as to why this mechanism was introduced as a method of control to maintain proper deoxynucleotide ratios for synthesizing DNA.

The first allusions to the asymmetric association of α2β2 came about from a \textsuperscript{1}H and \textsuperscript{19}F nuclear magnetic resonance (NMR) study utilizing deoxyuracilose-5′-diphosphate as a spectroscopic probe where it was found that the number of binding sites on the α2 subunit was halved upon association with the β2 subunit [22,23]. Decades later, macromolecular analysis provided strong evidence that the protein oligomerization state directly regulates activity, where an inactive octamer α4β4 complex forms in the presence of dATP or ATP in combination with dTTP or dGTP [24]. However, the presence of all four substrates convolutes the enzyme oligomer state and can result in inhibited α/β complexes with varying ratios.

The asymmetric α2β2 model revealed by the cryo-EM structure appears to represent the major association mode between the two subunits during catalysis. This updated model inspires new insight into half-of-sites reactivity where the asymmetrical subunit interaction mode better accommodates all four types of substrates in the α subunits. The benefit of asymmetric interaction lies in its allowance that (1) the two active sites of α2 do not necessarily have to bind substrates simultaneously and (2) distinct substrates can bind to the two active sites in the α2 homodimer. Since the four types of substrates, ADP, GDP, CDP, and UDP, may have a discernible difference in binding and catalytic rates, the probability of having the active sites at α2 simultaneously loaded with identical substrates is a small population of the total.

It is worth mentioning that the asymmetric subunit interaction mode enables a substantial degree of flexibility that eliminates the need for substrate loading at both α2 active sites. This is a unique need as the catalytic competition for the complex consists of two proteins, and the transient protein complex must handle multiple types of substrates. The most likely event would be that one α subunit is bound with the substrate and causes an asymmetric interaction of the β subunit. This interaction would support half-of-sites reactivity in terms of the number of tyrosyl radicals engaged (maximally two per β2) and transferring to the α2 subunit. In essence, all tyrosyl radicals in the two β2 sites are catalytically competent but are not necessarily involved in catalysis.
simultaneously. To place RNR in a biologically relevant context, regulation of in-cell pools of NTPs/dNTPs is critical in DNA replication, cell cycle progression/proliferation, and biomass production. Recently, excess levels of individual nucleotides were found to result in the inhibition of cell proliferation and that cell growth and cell division can therefore be decoupled due to these imbalances [25]. As a crucial enzyme, RNR must be able to handle in-cell changes due to environmental stressors, such as nutrient imbalances, and has developed a very complex half-sites method with the use of substrate and effector sites and a highly transient but efficient catalytically active complex to maintain proper physiologically relevant levels of dNTPs.

While the asymmetric interaction supports half-of-sites reactivity, the less optimized radical content, often in the range of 1.2–2 Tyr/β2 subunit in E. coli RNR, adds additional complexity to the perceived less optimal reactivity of the enzyme. The tyrosyl radical is self-assembled, requiring Fe$^{2+}$ and oxygen. Although the tyrosyl radical in the enzyme exhibits an extraordinary stability in the as-isolated protein, it has been reported in the past to contain ~60% of the radical content than what is expected in the as-isolated E. coli RNR β2 subunit [13,14,26]. Unlike the α2 subunit, which requires an external reductant to undergo multiple turnovers, the tyrosyl radical of the β2 subunit is regenerated immediately after catalysis, ready for more action with other α2 subunits [27]. This begs the question as to why the expression of β2 does not result in the expected 2 Tyr radicals/β2 and what role metallocofactor assembly plays in this reduced usability of β2 [28,29]. The method of metallocofactor assembly and metal loading in vitro is still unknown, though an in vitro study shows that there are 1.2 Tyr radicals/3.6 iron ions [30]. To begin addressing this question, a recent in-cell electron paramagnetic resonance (EPR) and double electron-electron resonance (DEER) study compared the in vitro radial distance to in-cell distance by overexpressing apo-β2, saturating the cells with oxygen, and measuring the mean distance between Tyr122’-Tyr122’ in the homodimeric protein which was found to be nearly identical, indicative of a similar and rigid radical environment for in-cell compared to in vitro RNR [31]. However, bulk spin concentration in-cell (~20 μM) is nearly an order of magnitude lower than in vitro samples (~200 μM) and in-cell radial distribution resulted in a lower-than-expected value compared to in vivo. The authors posit that the reduced value of in-cell radial distribution possibly accounts for the presence of one Tyr/β2 subunit, which aligns with the asymmetric binding observed in cryo-EM but conflicts with the “two radicals or none” concept described for in vitro studies. However, they admit that these results are still preliminary. This in-cell study suffers from some pitfalls regarding the quantitation of the Fe cofactor incorporated into the β2 subunit compared to the tyrosyl radical detected. There is a possibility that full incorporation of the cofactor is disrupted, resulting in the low radical concentration detected. There is also a possibility of apo-β2 concentration being much lower than expected. The cellular environment introduces many variables that need to be considered to reliably compare in-cell radical concentration to those well-characterized values of in vitro studies.

**Prostaglandin endoperoxide synthase**

The isozymes prostaglandin endoperoxide synthases 1 and 2 (PGHS) have been observed to behave as conformational heterodimers with allosteric and catalytic subunits throughout catalysis [32–36] (Figure 2). PGHS enzymes catalyze the conversion of the ω-6 polyunsaturated fatty acid arachidonate (AA) to prostaglandin H$_2$ (PGH$_2$) (Figure 2a) by utilizing a heme-dependent peroxidase (POX) site to generate a tyrosyl radical and later reduce prostaglandin G$_2$ (PGG$_2$) to PGH$_2$, and a cyclooxygenase (COX) site for controlled stereospecific free-radical oxidation, introducing five chiral stereocenters in the product [37–39]. While they both catalyze the production of PGH$_2$, the isozymes are found in different tissues. They are attenuated for separate physiological pathways, as reflected in their differing allosteric responses to fatty acid substrate and inhibitor binding [37,40–42].

PGHS proteins have been known for decades to be homodimers. The monomer form is not catalytically active, and the monomers of each dimer were first thought to work separately [40]. From a wealth of accumulated data, including crystallographic, mutagenic, and functional studies, it is now understood that subunits work together to function as cooperating heterodimers in solution [32,43]. As a fully functioning homodimer has not been observed, these enzymes may employ half-of-sites reactivity to regulate the turnover of their substrates. Nonequivalence of the PGHS subunits is supported by stoichiometric analysis of heme incorporation. First observed in titration studies [44], both monomers have high-affinity metalloporphyrin sites but differ slightly in heme affinity. Both isoforms reach maximum COX activity with one heme per dimer, suggesting that the POX site of one monomer must be active for COX activity. Further, mutation studies determined that rendering the POX site of one monomer unable to bind heme did not impair turnover [44,45]. It has been found that although dimerization is required for catalysis, PGH2 turnover only requires heme to attach to the high-affinity site per dimer. Moreover, the high-affinity heme monomer is the catalytic monomer [35,41]. Evidence suggests that the partnering subunits are homodimers prior to ligand binding (Figure 2b) [40,41]. Monomers of PGHS-2 are observed to bind various substrates with inequivalent orientations, only binding one substrate in the catalytically productive orientation. While this AA binding inequivalence is not observed in PGHS-1 crystal structures, some NSAIDs...
cause inhibition of PGHS-1 by binding the COX site of only one monomer [35, 36, 40–42].

Allosteric regulation between inequivalent COX sites implies communication across the tightly associated dimer interface and cross-linking studies point to ligand-dependent crosstalk at the interface. However, the subtlety of this response remains unknown [40, 41]. Structural data show a network of residues in contact with an essential arginine sensitive to ligand binding in the COX active site [42]. In the binding pocket of PGHS-1, interactions between arginine and tyrosine residues and the carboxylate of fatty acid ligands induce movement such that the monomer takes on one of two conformations and becomes either catalytic or allosteric [35, 36, 41]. In explanations put forward, the nature of the substrate bound in the COX site of the allosteric monomer dictates catalytic activity [34, 35]. When this site is unoccupied, the catalytic activity in the partner monomer is inhibited. Most fatty acids, even non-substrates, can bind to the allosteric site. The variation of binding modes attained to accommodate diverse fatty acid ligands is reflected by distinct catalytic responses to allosteric inhibition and stimulation. Ligand-induced movement could elicit changes at the dimer interface that modulate COX activity at several junctions, such as increasing AA binding selectivity, stabilizing the tyrosyl radical, or tuning AA orientation to alter hydrogen abstraction [46]. However, the role of the substrate orientation relative to these residues in PGHS-2 is not well understood.

Given that PGH2 does not accumulate in tissue but is rapidly shuttled through a cascade of downstream biochemical processes, nature may employ half-of-sites reactivity to regulate its synthesis. Notably, a PGHS1/2 heterodimer has been shown to be feasible and may demonstrate allostery as well [47]. Using substrate-induced nonequivalent subunits, PGHS enzymes can respond kinetically with additional means to factors like AA concentration and other fatty acids to modify PGH2 production [34].

**Other enzymes that exhibit half-of-sites reactivity**

While we have focused on two examples of half-of-sites reactivity in oxygen-dependent enzymes, this concept may be more broadly applicable than previously thought.
Tryptophan 2,3-dioxygenase (TDO), recently reclassified as part of the heme-dependent aromatic oxygenase superfamily [48], is an oxygen-dependent enzyme in the kynurenine pathway for tryptophan degradation and *de novo* NAD⁺ biosynthesis in mammals. The quaternary structure of the enzyme is arranged as dimer-of-dimers, with each monomer subunit containing a heme binding site and substrate binding site adjacent to the heme in its distal pocket [49–52]. Spectroscopic characterization of TDO brings to light the presence of two electronically inequivalent ferric hemes upon the addition of L-Trp [53–55]. Cooperative binding of L-Trp to human TDO has been experimentally established via resonance Raman spectroscopy [56]. These inequivalent hemes beg the question of the type of advantage this intrinsic characteristic imparts to regulating catalysis, which may be due to the cooperative binding of organic substrate or O₂ (heme ligand) increasing binding affinity for the other heme site. If this is the case, the likely purpose is to tune reactivity according to high or low L-Trp levels, though further study is necessary.

**Figure 3**

Structure of ACMSD. (a) The first image is a side-by-side comparison of the zinc center found in the homodimer structure, and the following image is an overlay of the two active sites. Observable differences in the positioning of metal–ligand His177 and the acid-base catalyst His228, where one site is more ordered around the metal center than the other [57,59]. (b) The size exclusion chromatography-small angle X-ray scattering (SEC-SAXS) tetramer solution structure shows that the dimer-and-dimer associates in a “head-on” manner with each active site containing two catalytically essential arginine residues (black arrows) with one of them (Arg239) coming from an adjacent subunit [58,60]. ACMSD, ω-amino-β-carboxymuconate-ε-semialdehyde decarboxylase; SAXS, small-angle X-ray scattering.

This phenomenon is also observed in ω-amino-β-carboxymuconate-ε-semialdehyde decarboxylase (ACMSD), which is part of the TDO-initiated kynurenine pathway for tryptophan catabolism. The two active sites of ACMSD differ, with one more ordered and the other more disordered (Figure 3) [57]. In the first-coordination sphere, the metal–ligand His177 loses contact and shifts away from the metal ion (4.2 vs. 2.2 Å). In the second coordination sphere, the acid–base catalyst His228, which assists in generating metal-bound hydroxide to drive catalysis, is more distant (4.5 vs. 3.3 Å) from the metal ion. This enzyme requires two Arg residues for substrate binding, one from a neighboring subunit [58,59], rendering the monomeric form catalytically incompetent. The enzyme is present in monomeric, dimeric, and higher-order oligomeric states, and the presence of substrate drives it to higher-order, catalytically more active forms [58,60]. Half-of-sites reactivity is concretely observed as a regulatory mechanism for enzymatic activity in this system in biochemical analyses [58]. ACMSD uses half-of-sites to add a layer of fine-tuning to regulate its
catalytic activity to meet fluctuating metabolic demands in a primary metabolic pathway. A dynamic ratio of catalytically inactive monomer, catalytically active but not fully optimized homodimer, and fully active high-order oligomeric states would meet the levels of tryptophan availability.

Cytidine triphosphate synthase (CTPS), which catalyzes the final step of de novo CTP biosynthesis, was suggested to have half-of-sites reactivity in the early 1970s. The affinity label, 6-diazo-5-oxo-norleucine, binds to half of the subunits but abolishes all glutamine activity of the enzyme [61]. Very recently, a cryo-EM structure was solved, which shows the precise location of all ligands in the CTPS structure and allows for a deeper understanding of how each ligand, i.e., GTP, UTP, and ATP, binding affects conformational changes in the structure [62]. Glyoxylase I, which converts a hemithioacetal, such as glutathione, and an α-ketoaldehyde into a thioester product [63], is a zinc-dependent homodimer that contains two active sites formed by contributions from each subunit [64–66]. NMR studies show that the active sites are not equivalent, consistent with the biochemical data [67,68]. Other enzymes, such as malate and lactate dehydrogenase, share half-of-sites characteristics. These are examples of a vast array of enzymes that could carry this half-of-sites reactivity concept to regulate their activity based on catalytic need or tune their reactivity to maintain delicate product pools.

Conclusion
Here, we have discussed several complex enzyme systems that present a half-of-sites reactivity phenomenon. These enzymes are functional dimers or high-order structures with identical primary structures, either with equivalent sites but not simultaneously in action or nonequivalent active sites. The relevant enzymes utilize this half of the active sites as a means of controlling catalytic activity as needed, such as in RNR, or as a method of added regulation to handle low but steady levels of product to protect from potential disease states, as observed in the case of ACMSD. While the term “half” in half-of-sites would usually dictate that approximately 50% of the active sites are active, this is not always the case. In some events, such as RNR, roughly 60% of the tyrosyl radical engagement is generally referred to, indicating that both active sites have the potential of being catalytically competent but are not always present. Still, its action is driven by the asymmetric binding of the two homodimer subunits to construct the radical transfer pathway and shuttle one radical from the β subunit to one α subunit active site. Previously, it was unsure whether half-of-sites reactivity in RNR results from in vitro reconstitution or non-optimal cellular conditions. The asymmetric binding mode of the subunits predestinates that only one of the tyrosyl radicals in RNR is active. Since the ribonucleotide reduction activity is controlled in part by the physical association of the subunits to allow radical transfer to the active site cysteine in the α subunit and return to the original tyrosine site in the β subunit after catalytic turnover, and each type of ribonucleotide has a different catalytic rate, a symmetric binding, and a simultaneous 100% of radical utilization cannot serve the biological purpose for RNR to handle various kinds of the substrates effectively. Thus, we posit that this method of governing catalysis is not an act of wasting resources but rather a natural strategy devised to accommodate the requirement for both high- and low-activity level needs within a single system dependent upon external factors. Substrate-binding induced inequivalent enzyme active sites often result from cooperativity among subunits. Those enzyme-active sites with residues from a neighboring subunit deserve attention for further study of their oligomerization state. The half-of-sites reactivity is not a poor evolutionary result; each case has a high biochemical rationale. As highlighted here, the latest progress has revealed exciting new understandings. Future studies will help build a deeper understanding of this phenomenon.

Declaration of Competing Interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability
No data was used for the research described in the article.

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Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest


15. Climent I, Sjöberg BM, Huang CY: This is a recent comprehensive review of advances in ribonucleotide reductase studies.


This EPR study provided an in-depth understanding of the PET involving radical transfer fragment between Tyr358* (β) and Tyr731* (α) across the α/β interface.


This paper describes the importance of regulating in-cell pools of NTP/dNTP, which is vital in DNA replication, cell cycle progression, and proliferation.


This article summarizes the mechanism of ribonucleotide reductases from a radical chemistry perspective and pointed the way to future frontiers in radical enzymology.


This work uses multi-frequency EPR, ENDOR, and DEER to characterize tyrosyl radical’s structure and electrostatic environment in cells, including site-specifically substituted 2,3,5-F3-tyrosine.


This review presents a thorough summary of structural evidence for cycloxygenase allostery.


This article details findings from recent mutational and structural studies that support crosstalk between monomers of COX homodimers and provides a kinetic model of allosteric interactions.


This article defines a new heme-dependent oxygenase family.


