All-atoms Simulations Disclose How Cytochrome Reductase Reshapes the Substrate Access/Egress Routes of its Partner CYP450s

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Abstract
Cytochromes P450 enzymes (CYP450s) promote the oxidative metabolism of a variety of substrates via the electrons supplied by the cytochrome P450s reductase (CPR) and upon formation of a CPR/CYP450 adduct. In spite of the pivotal regulatory importance of this process, the impact of CPR binding on the functional properties of its partner CYP450 remains elusive.
By performing multi-μs-long all-atoms molecular dynamics simulations of a 520,000-atoms-model of a CPR/CP450 adduct embedded in a membrane mimic, we disclose the molecular terms for their interactions, considering the aromatase (HA) enzyme as a proxy of the CYP450s family.
Our study strikingly unveils that CPR binding alters HA's functional motions, bolstering a change in the shape and type of the channels travelled by substrates/products during their access/egress to/from the enzyme's active site. Our outcomes unprecedentedly contribute to extricate the many entangled facets of the CYP450s metabolon, redrafting its intricate panorama from an atomic-level perspective.

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Cytochrome P450 enzymes (CYP450s) intervene in many vital biological functions such as the metabolism of endogenous/exogenous substrates and the biosynthesis of steroid hormones.[1] In

particular, the aromatase enzyme (HA, alternatively named CYP19A1) promotes the conversion of androgens to estrogens thanks to the electrons[2,3] supplied by the cytochrome P450s reductase (CPR), a transmembrane protein, which satisfies the electrons needs of many distinct CYP450s (i.e. CYP11A1, 17A1, 2C9, 3A4, 2D6). Featuring the human CYP450s isoforms a similar fold, an alike complexation and electronic transfer (ET) mechanism is also likely.

At the endoplasmic reticulum (ER) surface, the CPR is stoichiometrically disfavoured with respect to its many distinct CYP450s partners (ratio is of 1:5-20). Hence, being CPR/CYP450s complexes functional in a 1:1 ratio, the CPR protein must overcome this stoichiometric imbalance to efficiently perform its function,[4] promptly dissociating from its redox partner after ET has occurred.

CPR performs this pivotal function thanks to its plastic architecture, composed by the nicotinamide adenine dinucleotide phosphate (NADPH), the flavin adenine dinucleotide (FAD) and the flavin mononucleotide (FMN) domains, which are joined by flexible loops. An additional loop connects the FMN domain to a trans-membrane helix. Crystal structures[5] and molecular dynamics (MD) simulations[6] entrenched the existence of CPR in a 'closed' conformation, where the three domains are in close contact, allowing an internal ET (from NADPH to FAD and, ultimately, to FMN cofactors), and in an 'open' conformation, where only the FAD and NADPH domains interact with each other (Figure S1 of the Supporting Information (SI)). Since the FMN domain ultimately delivers electrons to CYP450s, CPR must interconvert from the 'closed' to 'open' state to promote ET.

Due to the peculiar CPR's conformational complexity and the scarcity of CPR/CYP450s adduct's crystal structures, MD simulations as of yet characterized only the binding of the FMN domain to CYP450s.[7,8] Starting from our previous work, where the FMN/HA model was obtained on the basis of a consensus protein-protein docking approach,[8] here we built and characterized the adduct between HA and the complete CPR protein in its open conformation (Figure S1) aiming at dissecting how CPR binding affects the structural, dynamical, and functional traits of its partner protein. To this aim, 3.5 μs-long MD simulations (for details see the Supplementary Methods in SI) were performed on the CPR/HA adduct. Three replicas of the system, named hereafter as CPR/HA$_{a/b/c}$, were run to support the reliability of our findings (Figures S2-S3).

As a result, the Hydrogen (H)-bonds analysis of the CPR/HA adduct (Table S1) highlights the presence of persistent salt bridges between the HA's basic (e.g. Lys420 and Lys352) and CPR's acid (e.g. Glu95 and Asp157) residues, the latter mostly belonging to FMN and NAPDH domains. This is consistent with mutagenesis and previous MD simulation studies, showing that CYP450's basic residues are critical for the interaction with the acidic CPR's residues.[8,9] Furthermore, the FAD and NADPH domains push the FMN domain towards the HA's heme proximal cavity, allowing the formation of additional H-bonds as compared to the FMN/HA adduct previously simulated[8] (Table S1). This scenario depicts an entangled topological network contributing to the formation of an ET prone adduct. In spite of the electrostatic complementarity of the HA/CPR interface, the calculated binding free energy and its components (Table S2) reveal that the hydrophobic interactions (Table S3) mostly drive the adduct's formation. This is consistent with CPR's need of establishing effective, yet transient, interactions with CYP450s to rapidly switch among its many redox partners, while the few salt bridges may be instrumental for their initial recognition. Our simulations also disclose that distinct membrane compositions (Table S4), differently affect the inclination and the immersion of HA towards/in the membrane, similarly to other CYP450s.[10]

Finally, in order to detect possible allosteric communication channels between the HA's active site and the proximal cavity,[7] we also compared the structures of the catalytic pocket in the presence and in the absence of the HA's substrate, androstenedione (ASD) (Figure 1A). Remarkably, we observed that ASD removal from the active site triggers specific conformational changes of the residues lining the proximal cavity. Namely, Arg115, initially H-bonding with the carboxyl groups of the heme (defined as Tail 1 and Tail 2) and the 17-carbonyl of ASD (Figure 1B), rearranges to fill the empty cavity. Conversely, Tail 2 loses its interaction with Arg115, while establishing a salt bridge with Arg145. The displacement of Arg145 in the proximal cavity, due to electrostatic repulsive

interactions originated from its approach to Lys440, triggers a remodelling of Tyr441 (Figure 1C). This affects the binding of FMN and, may ultimately influence the ET process. This conformational remodelling exclusively occurs upon ASD removal in the CPR/HA adduct, while being absent in the uncomplexed HA.

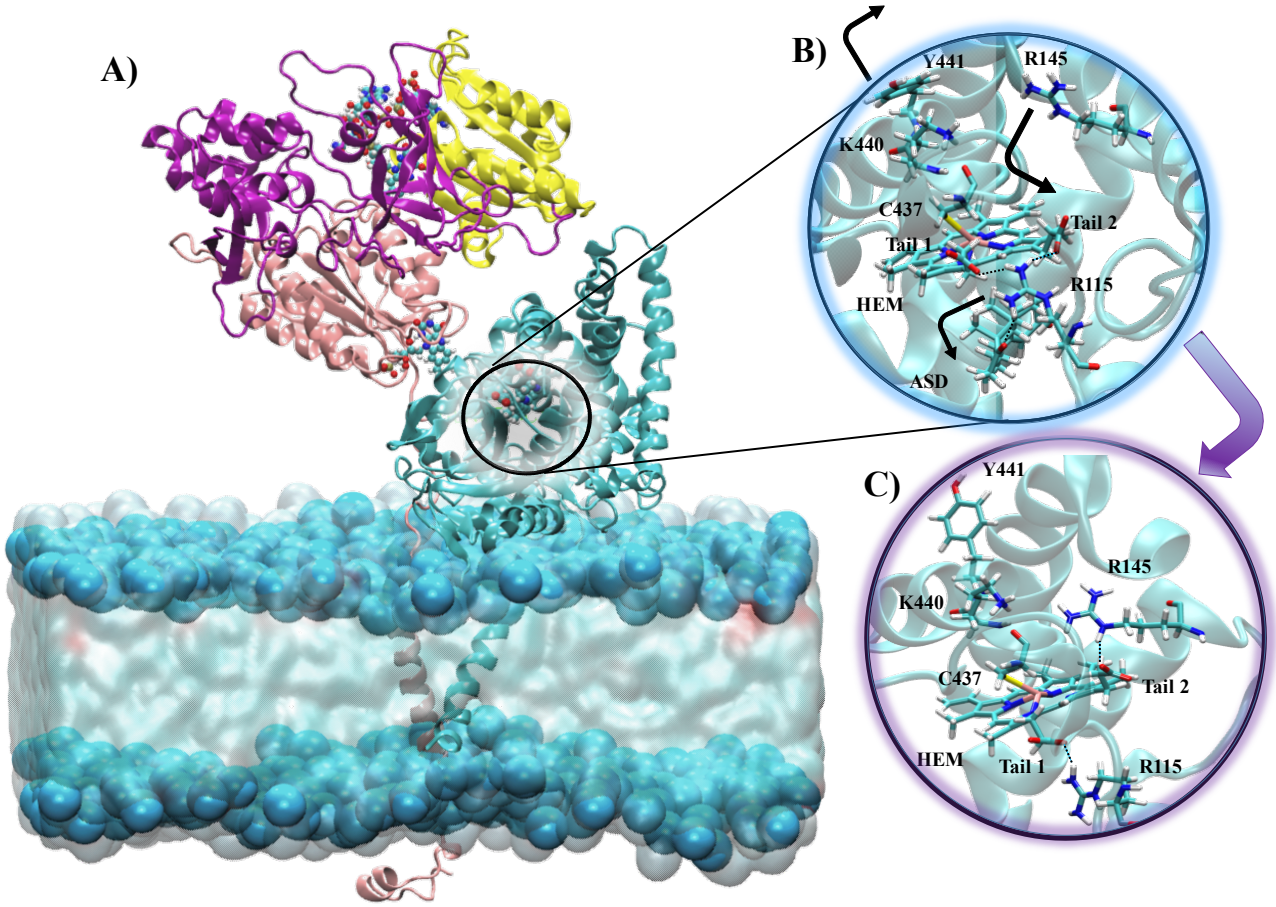

Figure 1. (**A**) Representative structure of the aromatase (HA) enzyme in complex with CYP450 reductase (CPR) extracted from a molecular dynamics trajectory. HA, flavine mononucletide (FMN), flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide phosphate (NADPH) domains are shown as cyan, pink, purple and yellow ribbon, respectively, while the 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine molecules, forming the membrane, are shown as transparent surface with phosphorus atoms highlighted as light blue van der Waals (vdw) spheres. Heme, FMN, FAD and NADPH cofactors are shown in vdw spheres. Structure of the heme and proximal cavities in the CPR/HA adduct in the presence (**B**) and the absence (**C**) of androstenedione. The black arrows indicate the movement of R115, R145 and Y441 passing from (**A**) to (**B**).

HA, alike all CYP450s, is characterized by a deeply buried active site, which is connected to the protein surface by multiple distinct impervious channels, representing the entry/exit routes of substrates, products and inhibitors.[11] Identifying those channels especially within a complex system like CYP450s is a difficult task.[12] Nevertheless, different studies dissected their types and roles in the CYP450 metabolism.[13-16] Due to the pivotal importance for CYP450's function, we inspected how CPR binding to HA reshapes these entry/exit routes.

As a result, in all replicas of the CPR/HA adduct, we identified two channels (Figure 2A) departing from the iron-atom. The first (labelled ChA) ends towards the membrane interface (Figure S4, Tables S5-S6 for the topological definitions of the channel) and corresponds to channel 2b of the general

CYP450's nomenclature,[11] suggested to be the substrate's entry route.[13,17] The second channel (named ChB) (Figure S4, Tables S5-S6) matches with channel 5[11] and is most probably travelled by water molecules and products.[18] Conversely, in uncomplexed HA (Figure 2B) we pinpointed three channels: two corresponding to ChA and ChB of the adduct, and a third one (ChC), exclusively present in HA, (Figure S4, Tables S5-S6). The latter matches with channel 2a of the CYP450's nomenclature,[11] which our previous study suggested to be the most likely exit route for HA's substrate and inhibitors.[13] This remarkable finding discloses that CPR binding triggers a conformational remodelling of HA's structure, causing ChC's closure.

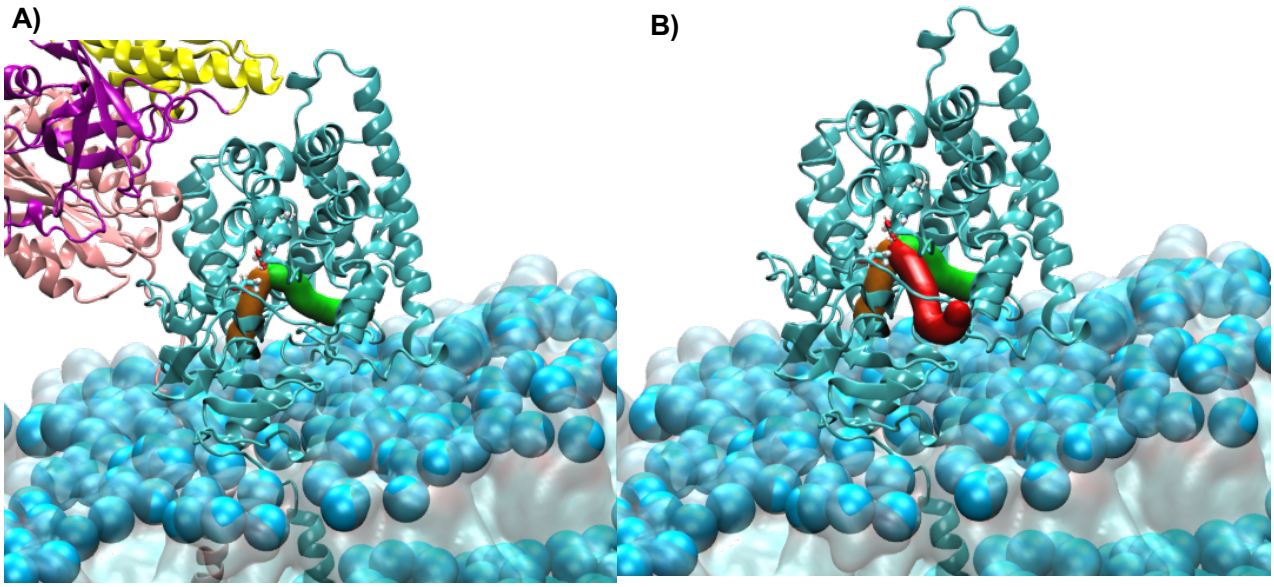

Figure 2. (**A**) The entry/exit channels to/from the catalytic site of the CPR/HA adduct (ChA and ChB, in green and orange QuickSurf representation, respectively). (**B**) Channels observed on uncomplexed HA (ChA, ChB and ChC are shown in green, orange and red QuickSurf representation, respectively).

Next, we computed the cross-correlation matrix (CCM) of CPR/HA and of HA to capture the dynamically coupled motions occurring within the system (Figures S5-S6). This analysis unequivocally reveals that HA positively correlates with the FMN, moving lockstep, while being anti-correlated with the remaining CPR's domains. By comparing the CPR/HA matrix with that of HA, it appears that the internal positive correlation of the enzyme increases upon CPR binding (Figure 3A). Additionally, the essential dynamics of the first principal component 1 (PC1) (Figure 3B) pinpoints a functional movement where the FMN domain and HA cooperatively push the FAD and NADPH portions of CPR. This promotes a conformational rearrangement of CPR, possibly leading it in a state prone to deliver electrons to the FMN domain, eventually preparing the FMN for the subsequent ET to HA.

The conformational subspace defined by PC1 vs PC2, which both strongly contribute to the variance (Figures S7-S8), reveals two major regions visited along the MD trajectory. The analysis of two most representative configurations, as extracted from a cluster analysis of these two regions, enlightens the molecular terms of HA's channel reshaping triggered by CPR binding. Namely, ChC's closure (Figure 3D) is due to a conformational rearrangement of the hydrophobic patch formed by Ile89, Phe116, Ile229, Met374. Ile89's remodelling triggers the rotation of Ile229 and Met374, inducing the formation of an H-bond between His62 and the carbonyl oxygen of Ile229 placed on the A' and F-helices, respectively. Stunningly, CPR binding even widens ChA due to a loss of hydrophobic and electrostatic interactions between Arg192, Gln218, Phe221, Gln225, and His480. Indeed, the breakage of the H-bond between Gln225 and the CO@Phe221 in helix-F and of the π- π interaction

between Gln218 and Arg192 causes the rotation of Gln225 and Gln218 (Figure 3F). Hence, CPR binding may facilitate the uptake of HA's substrates into the active site, creating a wider and, thus, energetically more viable entrance route.
Additional analyses derived from the CCM further confirm the role of these secondary structure elements in ChC (ChA) closing (opening) (Figure S9).

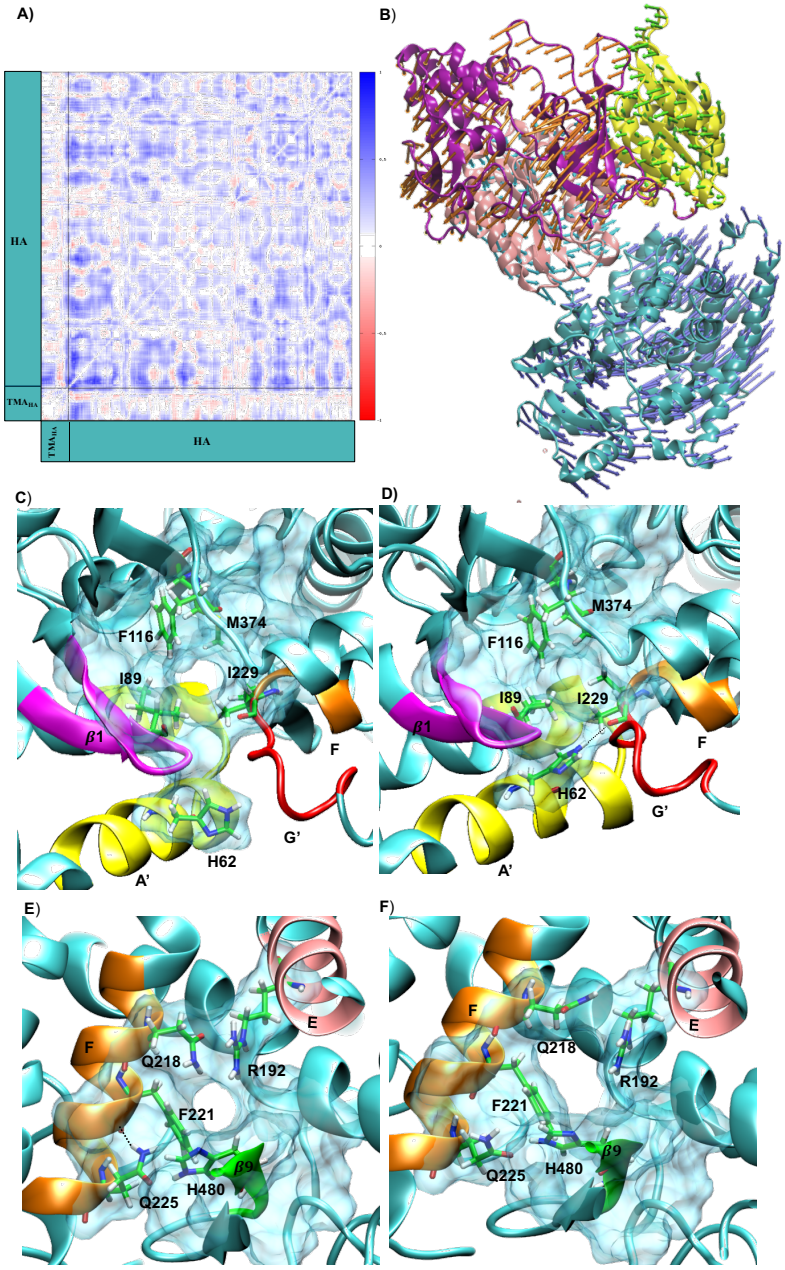

Figure 3. **(A)** Difference cross-correlation matrix calculated between the HA in the CPR-bound and free form. The protein names and their domains are reported on the bottom and on the left of the matrix. TMA_{HA} indicates the HA trans-membrane anchor. (**B**) Essential dynamics of CPR/HA adduct as revealed by principal component analysis. The arrows show the motion of C_α, C, O, N atoms. HA, FMN, FAD and NADPH domains depicted in cyan (violet arrows), pink (cyan arrows), purple (orange arrows) and yellow (green arrows) new cartoon representations, respectively. The transmembrane helices of both HA and the FMN domain are not shown for clarity. Representation of the open (**C**) and closed (**D**) channel C (ChC) and of the enlargement (**E**-**F**) of channel A (ChA). HA, A', β1, F, G' and E helices and β9 loop are shown in cyan, yellow, purple, orange and red, pink, and green new cartoon representations, respectively. The residues involved in the closure/opening of the

ChC/ChA, are represented in licorice.

In summary, our outcomes contribute to (i) disclose that CPR interacts with its partner CYP450s mostly by establishing hydrophobic interactions, besides few specific critical salt bridges, which may contribute to initial recognition complex. This is consistent with the CPR's need of establishing transient interactions with many distinct CYP450s to overcome its disfavoured stoichiometric imbalance; (ii) identify a possible pathway of allosteric communication between HA's catalytic site and the proximal cavity, where the FMN domain binds. This involves a remodelling of Arg115 triggered by ASD binding/dissociation to/from the CPR/HA adduct; (iii) unravel that CPR binding alters the substrate uptake/release routes and, possibly, the energetics associated to their channelling through HA, by triggering the closure of ChC, preferentially travelled by the substrate/inhibitor in the uncomplexed enzyme,[13] while inducing a widening of ChA, involved in the entry of the substrate from the membrane.
Hence, our study, contributes to disentangle the many facets of the CYP450's metabolon, providing an unprecedented picture of the impact of CPR binding on its partner CYP450. Being deregulated CYP450's activity implicated in distinct cancers' onset, this information supplies the molecular basis for rationally designing novel anti-cancer drug-candidates able to interfere with the formation of an ET functional CPR/HA adduct or possessing fine-tuned pharmacokinetic properties to optimally exploit the CPR-induced remodelling of the HA's entrance/egress routes.

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Supporting information. Computational Details, Figure S1 to S9. Table S1 to S6. This material is available free of charge at via the Internet at http://pubs.acs.org.

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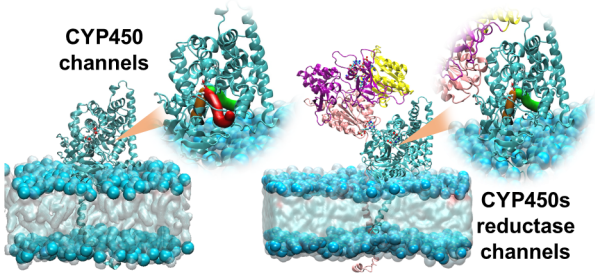
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