Archives of Biochemistry and Biophysics 606 (2016) 111–119

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Role of active site loop in coenzyme binding and flavin reduction in cytochrome P450 reductase

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ARTICLE INFO

Article history:
Received 2 May 2016
Received in revised form
20 July 2016
Accepted 22 July 2016
Available online 25 July 2016

Keywords:
Cytochrome P450 reductase
Stopped-flow spectroscopy
Flavin enzymology

ABSTRACT

Cytochrome P450 reductase (CPR) contains a loop within the active site (comprising Asp^{634}, Ala^{635}, Arg^{636} and Asn^{637}; human CPR numbering) that relocates upon NADPH binding. Repositioning of the loop triggers the reorientation of an FAD-shielding tryptophan (Trp^{679}) to a partially stacked conformer, reducing the energy barrier for displacement of the residue by the NADPH nicotinamide ring: an essential step for hydride transfer. We used site-directed mutagenesis and kinetic analysis to investigate if the amino acid composition of the loop influences the catalytic properties of CPR. The D634A and D634N variants elicited a modest increase in coenzyme binding affinity coupled with a 36- and 10-fold reduction in cytochrome c^{3+} turnover and a 17- and 3-fold decrease in the pre-steady state rate of flavin reduction. These results, in combination with a reduction in the kinetic isotope effect for hydride transfer, suggest that diminished activity is due to destabilization of the partially stacked conformer of Trp^{677} and slower release of $NADP^+$. In contrast, R636A, R636S and an A635G/R636S double mutant led to a modest increase in cytochrome c^{3+} reduction, which is linked to weaker coenzyme binding and faster interflavin electron transfer. A potential mechanism by which Arg^{636} influences catalysis is discussed.

1. Introduction

Cytochrome P450 reductase (CPR) is a 78 kDa membrane-bound diflavin oxidoreductase that is localized to the cytoplasmic side of the endoplasmic reticulum and the outer membrane of the nuclear envelope. The enzyme uses two non-covalently bound flavin cofactors, FAD and FMN, to catalyze the transfer of reducing equivalents from NADPH to numerous physiological acceptors, including cytochrome b_5, squalene monooxygenase, and heme oxygenase [1–3]. CPR also supplies electrons required by microsomal cytochrome P450 monooxygenases (CYP), which catalyze diverse oxidative reactions on numerous substrates including steroids, fatty acids, drugs, pesticides, and carcinogens [4,5]. Electron transfer in CPR initiates with donation of a hydride ion from NADPH to FAD to form the FAD hydroquinone ($FADH_2$) [6]. This latter intermediate subsequently shuttles single electrons to the higher potential FMN cofactor, which in turn donates electrons to an external redox partner [7,8].

CPR is a dynamic multi-domain enzyme that toggles between an extended and compact state in the process of transferring reducing equivalents from NADPH to external electron acceptors [9]. The first crystal structure of CPR depicts the enzyme in a compact conformation, with the N-terminal FMN-binding domain (a structural homologue of bacterial flavodoxin) docked within the concave surface of the C-terminal NADPH/FAD binding domain, which is structurally related to ferredoxin $NADP^+$-reductase [10,11]. Bifurcating the flavin binding domains is a connecting sub-domain that positions the FAD and FMN isoalloxazine rings in close proximity for rapid and direct electron transfer. Transition to an extended conformation, defined by separation of the flavin-binding domains, is required for delivery of electrons from the reduced FMN cofactor to the P450 heme centre. An extended conformer of CPR, one that is capable of electron transfer to P450s and deficient in interflavin electron transfer, was structurally captured in a yeast-mammalian CPR chimera and a variant of rat CPR in which four residues were deleted from the flexible hinge region [11,12].

Structural analysis of CPR also revealed that the enzyme undergoes a number of substrate-induced conformational changes leading up to the transfer of a hydride ion from NADPH to FAD [13]. In substrate-free structures of CPR, captured in a disulfide-linked rat CPR variant and a yeast-human hybrid, the entire indole ring

Abbreviations: CPR, cytochrome P450 reductase; MSR, methionine synthase reductase; 2′,5′-ADP, 2′,5′-adenosine diphosphate.

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http://dx.doi.org/10.1016/j.abb.2016.07.018
0003-9861/

of the penultimate Trp^{679} (human CPR numbering) lies planar to the *re*-face of the FAD isoalloxazine ring, shielding the cofactor from the solvent and blocking catalytic placement of the NADPH nicotinamide ring (Fig. 1) [12,13]. The solvated carboxylate side chain of a conserved Asp^{634} (originating from an adjacent loop that comprises residues Gly^{633} – Asn^{637}) projects into the coenzyme-binding cleft. Docking of NADPH into the binding cleft places the ribityl pyrophosphate group of the coenzyme in close proximity to the side chain of Asp^{634}. To avoid steric clash with the ribityl group and electrostatic repulsion from the pyrophosphate, Asp^{634} rotates towards the N-terminal end of an α-helix, forming hydrogen bonds with the backbone amides of Asn^{637}, Met^{638} and Ala^{639} [13]. As a consequence of this loop movement, Trp^{679} reorients such that only the phenyl portion of the indole ring makes van der Waals contact with the FAD. The reduced electronic overlap between the two ring systems presumably lowers the energy barrier for the final conformational step prior to hydride transfer: displacement of Trp^{679} from the re-face of the FAD by the nicotinamide ring. This latter step is essential for positioning the C4 atom of the nicotinamide ring close to the N5 of the FAD for hydride transfer. A crystal structure of CPR with Trp^{679} and Ser^{680} deleted supports this latter conformational switch as it shows the nicotinamide and FAD isoalloxazine rings within van der Waals contact, with a 30° tilt between the planes of the two rings [14].

Thus, interactions between the coenzyme and residues within the active site Gly^{633}– Asn^{637} loop, hereafter referred to as the Asp-loop, guide conformational changes that lead to hydride and interflavin electron transfer. Sequence alignment of several eukaryotic diflavin reductases and the reductase domain of flavocytochrome P450 BM3 reveal that the composition of the Asp-loop varies (Fig. 2A). For example, Asp^{634} is conserved in five of the six enzymes shown, with the exception of novel NADPH-oxidoreductase 1, which contains an Asn in this position. Flavocytochrome P450 BM3, which elicits high rates of NADPH hydride transfer, shows the highest sequence variability in this region [15]. In this enzyme, the neighbouring Ala^{635} is replaced with Gly (Gly^{1002}) and Arg^{636} is swapped for a Ser (Ser^{1003}). Given that members of the diflavin reductase family elicit different rates of hydride transfer, we were interested in determining if residues within the loop influence the kinetic properties of the enzyme. We therefore created the following single amino acid substitutions: D634A, D634N, A635G, R636A and R636S in human CPR. We also generated the following double mutant in human CPR A635G/R636S to recreate the Asp-loop observed in flavocytochrome P450 BM3. UV/vis spectrophotometry and anaerobic stopped-flow spectrophotometry were used to evaluate the kinetic behavior of each variant.

2. Materials and methods

2.1. Materials

The substrates cytochrome c^{3+}, NADPH, NADP$^+$, FAD and FMN were purchased from Sigma Aldrich (Oakville, ON, Canada). [4(R)-^{2}H]NADPH (A-side NADPD) was prepared and purified as previously described [16]. *Pfu* Turbo DNA polymerase and Xl1 Blue cell lines were obtained from Agilent Technologies (Mississauga, ON, Canada). Protein purification supplies and columns were purchased from GE Biosciences (Mississauga, ON, Canada). All other chemicals were purchased from VWR.

2.2. HPLC analysis of flavin content

The flavin content of the variants was quantified by HPLC following a protocol similar to that previously published [17].

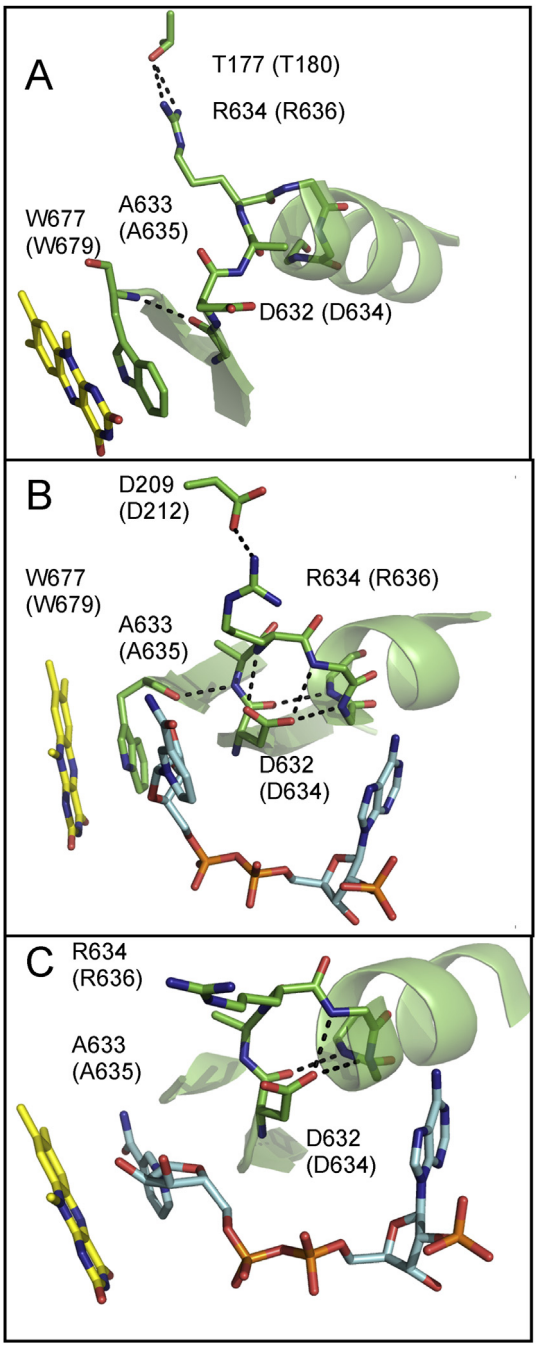

Fig. 1. Structural changes in the CPR active site upon coenzyme binding. Rat and human CPR numbering are presented with the latter shown in parentheses. Panel A depicts the NADP(H)-free form of CPR with the entire indole moiety of Trp^{679} in van der Waals contact with the FAD isoalloxazine ring and Asp^{634} (human CPR numbering) protruding into the active site. The illustration was generated from a disulphide cross-linked form of rat CPR (PDB entry 3OJW). Panel B illustrates the initial conformational changes that occur upon coenzyme binding. Electrostatic repulsion between the carboxylate side chain of Asp^{634} and the incoming pyrophosphate moiety of the coenzyme induces reorientation of Asp^{634} such that it forms hydrogen bonds with the backbone atoms of Arg^{636}, Asn^{637} and Met^{638} (PDB entry 1JA1). Trp^{679} also rotates such that only the phenyl portion of the side chain overlays with the FAD isoalloxazine ring. In this pre-catalytic state, the nicotinamide ring is projected away from the FAD isoalloxazine ring and towards the FAD adenine ring (not shown). Panel C depicts, the catalytically productive state, which was captured in a W677X variant of rat CPR (PDB entry 1JA0). In this structure, the nicotinamide ring lies against the *re*-face of the FAD isoalloxazine ring, with a 30° tilt between the two ring systems. CPR is in green cartoon, and the FAD isoalloxazine ring and NADP$^+$ are shown in yellow and cyan stick model, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)