Archives of Biochemistry and Biophysics 547 (2014) 18–26

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

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

Proximal FAD histidine residue influences interflavin electron transfer in cytochrome P450 reductase and methionine synthase reductase

Carla E. Meints, Sarah M. Parke, Kirsten R. Wolthers *

Department of Chemistry, University of British Columbia Okanagan, 3333 University Way, Kelowna, BC V1V 1V7, Canada

ARTICLE INFO

Article history:
Received 15 January 2014
and in revised form 17 February 2014
Available online 28 February 2014

Keywords:
Methionine synthase reductase
Cytochrome P450 reductase
Electron transfer
Flavin
Stopped-flow spectroscopy

ABSTRACT

Cytochrome P450 reductase (CPR) and methionine synthase reductase (MSR) transfer reducing equivalents from NADPH to FAD to FMN. In CPR, hydride transfer and interflavin electron transfer are kinetically coupled steps, but in MSR the two catalytic steps are represented by two distinct kinetic phases leading to transient formation of the FAD hydroquinone. In human CPR, His^{322} forms a hydrogen-bond with the highly conserved Asp^{677}, a member of the catalytic triad. The catalytic triad is present in MSR, but Ala^{312} replaces the histidine residue. To examine if this structural variation accounts for differences in their kinetic behavior, reciprocal substitutions were created. Substitution of His^{322} for Ala in CPR does not affect the rate of NADPH hydride transfer or the FAD redox potentials, but does impede interflavin electron transfer. For MSR, swapping Ala^{312} for a histidine residue resulted in the kinetic coupling of hydride and interflavin electron transfer, and eliminated the formation of the FAD hydroquinone intermediate. For both enzymes, placement of the His residue in the active site weakens coenzyme binding affinity. The data suggest that the proximal FAD histidine residue accelerates proton-coupled electron transfer from $FADH_2$ to the higher potential FMN; a mechanism for this catalytic role is discussed.

Introduction

Diflavin reductases are multi-domain enzymes that shuttle electrons derived from NADPH via two non-covalently bound flavin cofactors, FAD and FMN, to their cognate redox partner proteins. The prototypic member of this small enzyme family, cytochrome P450 reductase, is the redox partner for microsomal cytochrome P450 monooxygenases, which function in steroid, fatty acid and prostagladin metabolism as well as the detoxification of xenobiotic compounds [1]. A second eukaryotic family member, methionine synthase reductase, transfers an electron to the cob(II)alamin prosthetic group of methionine synthase, a process that leads to reductive remethylation of the vitamin B_{12} cofactor and restoration of methionine synthase activity [2,3]. Methionine synthase is vital for cell homeostasis as it generates tetrahydrofolate and methionine by transferring a methyl group from methyltetrahydrofolate to homocysteine [4]. Other members of the diflavin reductase family include novel oxidoreductase 1, and the reductase domains of nitric oxide synthase and P450 BM3, and the alpha subunit of bacterial sulfite reductase [5].

The diflavin reductase protein scaffold comprises three domains: an NADP(H) and FAD binding domain that is homologous to ferredoxin $NADP^+$-oxidoreductase (FNR),[1] an FMN-containing flavodoxin (Fld)-like domain, and a connecting domain that tethers the flavin domains and positions the cofactors such that their dimethylbenzyl moieties are <4 Å apart [6]. The reductive half-reaction initiates with hydride ion transfer from NADPH to the N5 of the FAD isoalloxazine ring. The FAD hydroquinone ($FADH_2$) subsequently shuttles single electrons to the higher potential FMN cofactor, which passes the electrons to a physiological redox partner protein [7]. The close juxtaposition of the FAD and FMN dimethylbenzyl moieties, depicted in the compact structure of rat CPR ostensibly allows for rapid interflavin electron transfer in CPR [6]. However, the compact conformation is not conducive for inter-protein electron transfer as the FMN cofactor is sequestered into the protein core. Thus, the enzyme is envisioned to transition to an extended conformation in which the two flavin domains are pried apart leaving the FMN solvent exposed and in position to interact with the electron acceptor protein. Several studies using a number of methodologies provide strong evidence that CPR

* Corresponding author. Fax: +1 250 807 8009.
E-mail address: kirsten.wolthers@ubc.ca (K.R. Wolthers).

[1] *Abbreviations used:* MSR, methionine synthase reductase; CPR, cytochrome P450 reductase; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; 2′,5′-ADP, 2′,5′-adenosine diphosphate; FNR, ferredoxin $NADP^+$-reductase; SVD, singular value decomposition; KIE, kinetic isotope effects; PDA, photodiode array; CT, charge-transfer.

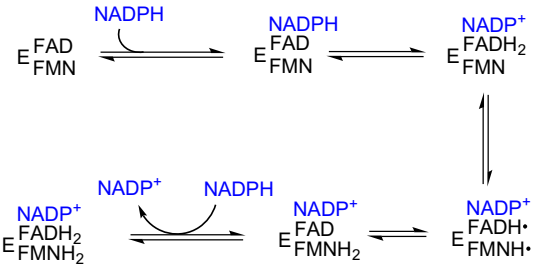
http://dx.doi.org/10.1016/j.abb.2014.02.009
0003-9861/

transitions between these two conformations during catalysis [8–12]. A 14-amino acid mobile hinge tethering the FMN-domain and connecting sub-domain in CPR has been shown to facilitate large-scale movement of the flavin domains with respect to each other [13]. Notably, the corresponding hinge region in MSR is considerably longer at 81 amino acid residues.

Stopped-flow spectroscopic studies following NADPH-reduction of fully oxidized CPR (E-FAD-FMN) showed that hydride transfer from NADPH to FAD is tightly coupled to interflavin electron transfer, such that there is no detectable formation of the E-$FADH_2$-FMN intermediate (Scheme 1) [14–16]. Reduction of CPR by the first mole of NADPH, represented by the first kinetic phase with a rate constant of ~30–60 s^{-1}, leads to transient formation of the two-electron form of the enzyme with 70% of the enzyme in the E-FAD-$FMNH_2$ state and the remaining 30% in the disemiquinone form (E-$FADH^{\cdot}$-$FMNH^{\cdot}$). The second slower kinetic event (3–6 s^{-1}) involves further bleaching of the flavin absorbance maxima at 454 nm and the disemiquinone signal at 600 nm as a population of the enzyme is reduced by a second equivalent of NADPH.

NADPH reduction of MSR is also multiphasic, but in contrast to CPR, the initial fast phase of flavin reduction involves formation of a charge-transfer complex at 120 s^{-1}, followed by hydride transfer at 20 s^{-1} [17]. Moreover, the spectral profile of the intermediate following this second kinetic phase is indicative of the E-$FADH_2$-FMN state of the enzyme. The third much slower kinetic phase (~1 s^{-1}) encompasses interflavin electron transfer, as it is associated with a small increase in the absorbance signal at 600 nm. Thus, in contrast to CPR, hydride and interflavin electron transfer are discrete kinetic steps, occurring on different time scales.

Intrigued by this difference in kinetic behavior, we compared the active sites of both enzymes. CPR and MSR possess three conserved active site residues, Ser^{460}, Cys^{632}, and Asp^{677} (human CPR numbering) in close proximity to the FAD isoalloxazine ring.



Scheme 1. Mechanism of NADPH reduction of a diflavin enzyme.

Mutagenesis of all three residues has established their catalytic importance for the CPR reductive half-reaction [18–20]. These residues are also present in plant-type ferredoxin $NADP^+$-reductases, with the exception that the asparate residue is replaced by a glutamate [21]. Fig. 1 shows that the local protein environment surrounding the conserved Asp is distinct in MSR and CPR. In CPR, the imidazole ring of His^{322} forms a hydrogen-bond with Asp^{677} (2.92 Å). A His residue is also coordinated to the conserved Asp carboxylate in the nitric oxide synthases, but in MSR, Ala^{312} replaces the imidazole ring, increasing the solvent exposure of the aspartate side chain [22,23]. Recent theoretical studies on FNR suggest that the conserved Glu carboxylate plays a key role in catalysis by acting as a proton donor to the N5 of the FAD isoalloxazine ring via the Ser side chain [24]. The corresponding residue in MSR and CPR may be serving a similar role, but in the reverse direction as a proton acceptor. Differences in the hydrogen bonding partners to Asp in MSR and CPR may affect the ability of the carboxylate to act as a general base, and this may account for differences in the kinetic behavior of the two enzymes. We therefore created reciprocal mutations of MSR and CPR in the form of a H322A variant of CPR and an A312H variant of MSR. We also inserted a glutamine into the active site in the form of a CPR H322Q variant and a MSR A312Q variant to preserve hydrogen-bonding capabilities to the Asp while eliminating acid/base functionality associated with the imidazole ring. A combination of stopped-flow and steady-state analysis was used to examine the affects of the substitutions.

Materials and methods

Materials

NADPH, $NADP^+$, 2′,5′-ADP, and cytochrome c^{3+} were purchased from Sigma–Aldrich. All other chemicals and reagents were purchased from Fisher Scientific. [4(*R*)-^{2}H]NADPH (A-side NADPD) was synthesized and isolated as previously described [25].

Expression and purification of MSR and CPR variants

The QuikChange site-directed mutagenesis kit (Agilent Technologies) was used to generate the CPR H322A, CPR H322Q, MSR A312H and MSR A312Q variants. The success of the mutagenesis was confirmed by DNA sequencing. The recombinant proteins were expressed in Rosetta2(DE3)pLysS strain of *Escherichia coli* and purified as previously described [26,27]. MSR and CPR concentrations were calculated from an absorbance reading at 454 nm using the extinction coefficients of 25,600 M^{-1} cm^{-1} and 21,600 M^{-1} cm^{-1}, respectively [26].

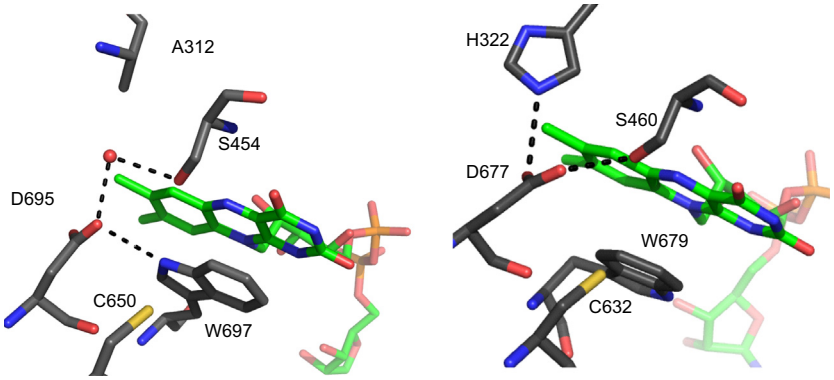


Fig. 1. The active sites of MSR (PDB ID: 2QTZ) and CPR (PDB ID: 3QE2), showing residues that comprise the catalytic triad surrounding the FAD cofactor (shown in green). Also shown are the different hydrogen-bonding partners to Asp^{677} of CPR and Asp^{695} of MSR. Hydrogen bonds are shown by black dotted lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)