Electron transfer in human cytochrome P450 reductase

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Abstract

Cytochrome P450 reductase (CPR) is a diflavin enzyme responsible for electron donation to mammalian cytochrome P450 enzymes in the endoplasmic reticulum. Dissection of the enzyme into functional domains and studies by site-directed mutagenesis have enabled detailed characterization of the mechanism of electron transfer using stopped-flow and equilibrium-perturbation methods, and redox potentiometry. These studies and the mechanism of electron transfer in CPR are reported herein.

Cytochrome P450 reductase (CPR) and its component domains

CPR functions as an electron bridge, transferring electrons between NADPH and the ubiquitous family of cytochrome P450 enzymes [1,2]. Cytochromes P450 are one of the most important families of proteins involved in the metabolic response of many living organisms to foreign chemicals [3]. The mono-oxygenation reaction catalysed by these haem-containing enzymes is dependent on a finely coupled and stepwise supply of electrons. CPR orchestrates electron supply from NADPH, an obligatory two-electron donor, to the P450 cytochromes, by stabilizing the one-electron reduced form of the flavin cofactors FAD and FMN. This stabilization gives rise to the blue semiquinone (sq) species (FADH+/FMNH−), which is observed in both kinetic and equilibrium studies of CPR.

The crystallographic structure of CPR suggests that the protein has evolved by gene fusion [4]. The NADPH/FAD domain is related to ferredoxin NADP-reductase, and the FMN domain has a strong structural resemblance to the flavodoxins [5–9]. An additional linker region of 125 residues connects the two flavin-binding domains. The linker is not involved in cofactor binding, suggesting that its role is predominantly structural. CPR is one of only four mammalian proteins known to contain both FMN and FAD in a single polypeptide chain. The isoforms of nitric oxide synthase [10], methionine synthase reductase [11] and protein NR1 [12] are the remaining members of the family. All are related structurally, but key differences also exist. For example, nitric oxide synthase binds calmodulin and has acquired additional domains and studies by site-directed mutagenesis have enabled detailed characterization of the mechanism of electron transfer using stopped-flow and equilibrium-perturbation methods, and redox potentiometry. These studies and the mechanism of electron transfer in CPR are reported herein.

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Key words: cytochrome P450 reductase, electron transfer, flavoprotein, kinetics. Abbreviation used: CPR, cytochrome P450 reductase; hq, hydroquinone; ox, oxidized; sq, semi-quinone.

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nitric oxide synthase \[20,21\], NADP\(^+\) release is thought to gate complete reduction of the flavin in the slower phase, consistent with a reversible scheme for flavin reduction in the isolated FAD domain and full-length CPR (Scheme 1). A number of the early kinetic phases are obscured in studies with full-length CPR \[17\]. This emphasizes the need for detailed study of single domains in complex redox enzymes.

In full-length CPR, the relatively high midpoint reduction potential of the FMN-ox/sq couple \(-66\ \text{mV}\) provides the driving force for inter-domain electron transfer \[16\]. This enables electron flow from NADPH to the cytochrome P450 enzymes (or other redox acceptors) and is a key aspect of the electron-transfer mechanism conserved in virtually all diflavin reductase enzymes \[13,14,22\]. Kinetic studies show that the transient accumulation of the blue disemiquinoid species of CPR occurs at a rate identical to that of hydride transfer from NADPH to FAD \[17\]. This indicates that inter-domain electron transfer is relatively fast. The blue disemiquinoid species subsequently decays following a second hydride transfer from NADPH (Scheme 1).

**Trp-676, coenzyme exchange and dual binding sites for NADPH**

Trp-676 in human CPR is positioned over the \(\text{re}\)-face of the FAD isooloxazine ring. The crystallographic structure of rat CPR indicates that the equivalent residue (Trp-677) would sterically prevent hydride transfer from NADPH to FAD in the absence of side-chain movement \[4,23\]. Rapid changes in tryptophan fluorescence emission accompany hydride transfer in the isolated FAD domain and full-length CPR \[17\]. Complex fluorescence transients are observed in full-length CPR, as two hydride equivalents are transferred to the enzyme. These fluorescence transients are diminished in a W676H mutant CPR, suggesting that Trp-676 is the origin of the fluorescence signal in the wild-type enzyme \[24\]. Exchange of Trp-676 for alanine compromises substantially the rate of hydride transfer in CPR, revealing a key role for the side chain of Trp-676 in enzyme reduction. The rate of FAD reduction in W676H CPR is modestly affected, but, importantly, the enzyme is reduced only to the two-electron level in rapid mixing experiments. Reduction beyond the two-electron level is prevented owing to the slow release of NADP\(^+\). This indicates a role for Trp-676 in the release of oxidized coenzyme from the FAD-binding domain. A double mixing stopped-flow method in which W676H CPR is reduced initially with stoichiometric NADPH, and following a suitable delay (100 ms) with excess NADPH, effects reduction to the four-electron level \[24\]. In the delay period, NADP\(^+\) can escape, albeit at a relatively low rate, prior to the binding of the second NADPH. In the direct mixing method, NADP\(^+\) becomes trapped in the binding pocket because a second NADPH binds rapidly to CPR and sterically prevents the release of NADP\(^+\) from the catalytic site. This complex model is consistent with a kinetic model (Scheme 1) that invokes the existence of two kinetically distinct binding sites for NADPH \[17\]. Only one site is catalytic; binding to the second site attenuates FAD reduction probably by interfering with NADP\(^+\) release from the catalytic site. In wild-type CPR, NADP\(^+\) release from the catalytic site is sufficiently rapid that binding to the second non-catalytic site does not prevent reduction to

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the four-electron level in direct mixing stopped-flow experiments with excess NADPH. Following the identification of two kinetically distinct coenzyme-binding sites in CPR and the unusual mechanism for electron transfer to FAD, similar dual binding-site models have been proposed for nitric oxide synthase [20] and the adrenodoxin reductase homologue FprA isolated from *Mycobacterium tuberculosis* [25].

The presence of two coenzyme-binding sites is unexpected, since they cannot be inferred solely from the crystal structure of CPR [4]. Kinetic studies with wild-type and W676H CPR at different concentrations of NADPH have, however, provided further support for the existence of two sites [17,24]. The rate of flavin reduction in the isolated FAD domain and in CPR increases as NADPH is decreased from molar excess to stoichiometric concentrations. At stoichiometric concentration, the second non-catalytic site is predominantly vacant and the partial inhibition of the rate of flavin reduction from the catalytic site is therefore relieved (Scheme 1).

Occupation of the non-catalytic site occurs at NADPH concentrations in excess of the enzyme concentration, and impairs NADP⁺ release from the catalytic site. This in turn partially inhibits flavin reduction, the rate of which is gated by NADP⁺ release. Pre-incubation of the enzyme with a stoichiometric concentration of adenosine 2′,5′-diphosphate does not lead to partial inhibition of the flavin reduction rate [26]. We infer that the binding of adenosine 2′,5′-diphosphate prevents NADPH from binding to the non-catalytic site. This observation also suggests that it is the NMN portion of NADPH bound at the second site that hinders NADP⁺ release from the catalytic site. Clearly, the control of CPR catalytic activity by coenzyme and fragments thereof needs to be re-evaluated in the light of these new kinetic data. Models that assume a single coenzyme-binding site for steady-state turnover to explain competitive inhibition by coenzyme fragments might be inappropriate. The effect of the second coenzyme-binding site on steady-state turnover and inhibition might lead to revised, and by necessity more complex, steady-state kinetic models.

**Relaxation kinetic studies of internal electron transfer in CPR**

A thermodynamic and kinetic framework for electron transfer in CPR has emerged from rapid mixing kinetic and potentiometry studies [16,17]. To access the kinetics of interflavin electron transfer we have employed temperature-jump relaxation kinetic methods, since these electron movements are difficult to monitor by the stopped-flow method. Relaxation kinetic methods bypass preceding steps that might limit the rate of inter-flavin electron transfer in rapid mixing approaches. Application of relaxation kinetic methods to
CPR has allowed direct measurement of the rate of inter-flavin electron transfer in enzyme reduced at the two- and three-electron level, and also opened up studies of conformational gating and ligand effects on this reaction [27].

A key requirement of the temperature-jump approach is to place the equilibrium of a reaction, such that perturbation of the equilibrium by rapid heating effects an absorption change that can be assigned to an individual reaction step (i.e. internal electron transfer from FAD to FMN in the case of CPR). This is especially difficult with CPR, where slow thermodynamic relaxation would lead to an equilibrium mixture of several different redox forms of the enzyme. The experimental conditions were selected carefully to minimize any uncertainty arising from the identity of the enzyme species in the final equilibrium distribution. Our potentiometry studies had indicated that in the two-electron-reduced enzyme the electrons are distributed as an equilibrium between the two enzyme species $\text{FAD}_{sq}/\text{FMN}_{sq}$ and $\text{FAD}_{hq}/\text{FMN}_{hq}$ (hydroquinone), and that $\approx50\%$ of each enzyme species is populated [16]. A rapid temperature increase shifts this equilibrium towards the $\text{FAD}_{sq}/\text{FMNH}_2$ form, and the kinetics of this process report on the rate of inter-flavin electron transfer [27] (Figure 2).

**Figure 2** | Temperature-jump transients for two-electron-reduced CPR

Transient a, NADPH-reduced CPR (1/$\tau$ = 55 ± 2 s$^{-1}$); transient b, dithionite-reduced CPR (1/$\tau$ = 11 ± 0.5 s$^{-1}$).

Adiabatic control of inter-flavin electron transfer

Inter-flavin electron transfer in CPR is relatively slow, and probably gated by an adiabatic process. For CPR reduced at the two-electron level with NADPH, two kinetic phases are seen in temperature-jump experiments [27]. Fluorescence and absorbance studies with dithionite-reduced CPR indicate that the fast phase (1/$\tau$ = 2200 ± 300 s$^{-1}$) is not associated with electron transfer, but is attributed to local conformational change in the vicinity of the FAD when CPR is reduced with NADPH. The slow phase (1/$\tau$ = 55 ± 2 s$^{-1}$) reports on internal electron transfer, $\text{FAD}_{sq}/\text{FMN}_{sq} \rightarrow \text{FAD}_{sq}/\text{FMN}_{hq}$. An intrinsic electron transfer rate of $\approx10^{10}$ s$^{-1}$ is predicted based on the separation distance of the two flavins in rat CPR [4,28]. The modest transfer rates observed in our temperature-jump studies leads us to conclude that electron transfer is gated. The reaction involves the deprotonation of the FAD blue sq, but solvent isotope effects do not accompany the reaction, ruling out a rate-limiting deprotonation reaction. Electron transfer is compromised in the presence of glycerol (75%, w/v), suggesting conformational gating [27]. A conformational search for geometries that maximize electronic coupling between the flavins is consistent with the atomic structure of CPR (and other diflavin reductases), which suggests a high degree of interdomain mobility [4,23,29].

**Electron transfer in the reverse direction and effects of Trp-676**

We have been able to poise the equilibrium distribution of enzyme species differently by reduction of CPR at the three-electron level [27]. Relaxation experiments with three-electron reduced CPR give access to the kinetics (1/$\tau$ = 20 ± 0.2 s$^{-1}$) of electron transfer in the non-physiological direction ($\text{FAD}_{sq}/\text{FMN}_{hq} \rightarrow \text{FAD}_{hq}/\text{FMN}_{sq}$). In the W676H mutant CPR, this rate is substantially increased (1/$\tau$ = 263 ± 3 s$^{-1}$), but the rate of electron transfer in the physiological direction ($\text{FAD}_{sq}/\text{FMN}_{hq} \rightarrow \text{FAD}_{sq}/\text{FMN}_{hq}$) is decreased by only a factor of $\approx2$ (1/$\tau$ = 27 ± 1 s$^{-1}$). This points to another important role for Trp-676 in controlling electron transfer in CPR. In addition to facilitating coenzyme exchange, this residue favours the transfer of electrons in the physiological direction (NADPH $\rightarrow$ FAD $\rightarrow$ FMN $\rightarrow$ haem).

**Coenzyme binding and inter-flavin electron transfer**

Although the initial equilibrium is the same, inter-flavin electron-transfer rates for CPR reduced by NADH (1/$\tau$ = 18 ± 0.7 s$^{-1}$) are $\approx3$-fold lower than those for CPR reduced by NADPH (1/$\tau$ = 55 ± 0.5 s$^{-1}$). Considering the redox and structural similarities between NADPH and NADH, the differences observed during temperature-jump experiments point towards a role for coenzyme binding in modulating inter-flavin electron transfer [26,27]. Further insight into the role of the 2′-phosphate of NADPH in enhancing inter-flavin electron transfer was obtained by performing temperature-jump experiments with coenzyme fragments and with dithionite-reduced enzyme [26]. The rate of inter-flavin electron transfer in dithionite-reduced CPR (1/$\tau$ = 11 ± 0.5 s$^{-1}$) is less than that for CPR reduced with NADPH (1/$\tau$ = 55 ± 0.5 s$^{-1}$). However, addition of 2′,5′-ADP to dithionite-reduced enzyme effects a $\approx3$-fold increase in the rate of inter-flavin electron transfer (1/$\tau$ = 35 ± 0.2 s$^{-1}$), and this is accompanied by a clear increase in the amplitude of the absorption signal change.
Thus the binding of the adenosine moiety of NADPH, and in particular the 2'-phosphate group, is a major factor in enhancing the rate of inter-flavin electron transfer. The binding of 2',5'-ADP does not perturb the midpoint reduction potentials of the four flavin couples, indicating that the enhanced electron transfer rates are not attributable to thermodynamic effects on electron transfer. We propose that ligand binding in the coenzyme-binding pocket effects a conformational change that optimizes coupling between the flavin cofactors.

Concluding remarks

Our studies with human CPR have illustrated the power of using a combination of approaches relying on (i) genetic ‘deconstruction’ of a complex redox enzyme, (ii) stopped-flow and relaxation kinetic methods and (iii) redox potentiometry to investigate the complex mechanism of electron transfer in this prototypical diflavin reductase. These methods are now being applied to other members of the diflavin reductase family.

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