Mechanism of cytochrome P450 reductase from the house fly: evidence for an FMN semiquinone as electron donor

Marat B. Murataliev*, René Feyereisen

Department of Entomology and Center for Toxicology, University of Arizona, Forbes 410, P.O. Box 210036, Tucson, AZ 85721-0036, USA

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Abstract The interaction of recombinant house fly (*Musca domestica*) P450 reductase with NADPH and the role of the FMN semiquinone in reducing cytochrome *c* have been investigated. House fly P450 reductase can rapidly oxidize only one molecule of NADPH, whereas the rate of oxidation of a second molecule of NADPH is too slow to account for the observed rates of catalysis. This demonstrates that house fly P450 reductase does not require a priming reaction with NADPH for catalysis. Kinetics of cytochrome *c* reduction and EPR spectroscopy revealed that the enzyme forms two types of neutral FMN semiquinone. One serves as the catalytic intermediate of cytochrome *c* reduction, and another one is an 'air-stable' semiquinone, which reduces cytochrome *c* 3000 times more slowly. The results show that the reduction state of the house fly P450 reductase during catalysis cycles in a 0-2-1-0 sequence.

Key words: Cytochrome P450 reductase; Flavoprotein; Flavin semiquinone; Catalytic mechanism

1. Introduction

Diflavin reductases, containing both FAD and FMN as prosthetic groups, are believed to have emerged as a fusion of the FAD-containing reductases (such as ferredoxin, b_5, or flavodoxin reductase) and flavodoxin, a small FMN-containing electron transfer protein [1]. This family of enzymes includes microsomal P450 reductases, the flavoprotein subunit of sulfite reductase, as well as the flavoprotein domains of natural fusion proteins such as NO synthase and P450BM3. The pathway of electron transfer in these enzymes is NADPH → FAD → FMN, and FMN serves as a one-electron donor.

Two mechanisms of electron transfer by the mammalian P450 reductase based on equilibrium redox potentials of the two flavins were proposed (see review [2]). Although these models differ in the maximal reduction state of the rat liver flavoprotein ($3e^-$ or $4e^-$), they have two common features. Both models require a priming reaction with NADPH, i.e. oxidized P450 reductase should be reduced by two molecules of NADPH (four electrons) before electron transfer from the FMN site can occur. Also, both models imply that FMN hydroquinone, but not semiquinone, serves as the electron donor. Mechanisms of electron transfer by other enzymes of the diflavin reductase family have either remained elusive or been assumed to be similar to that of mammalian P450 reductase.

However, recent studies show that redox potentials of the flavin cofactors of P450 reductases from various sources are significantly different from those of the rat liver P450 reductase [3]. The same conclusion has been reached for the properties of the flavins in the flavoprotein domain of P450BM3 [4]. Indeed, a detailed characterization of the P450BM3 catalytic mechanism demonstrated that, unlike the mammalian microsomal P450 reductase, P450BM3 does not require a priming reaction with NADPH, so that catalytic turnover can be completed at [NADPH] < [P450BM3]; maximal reduction state during catalysis does not exceed 2; and FMN semiquinone is the active catalytic intermediate [5].

An FMN semiquinone also serves as the catalytic intermediate of cytochrome *c* reduction by sulfite reductase [6], and has been proposed to reduce heme iron in nitric oxide synthase [7]. Moreover, an *Escherichia coli* flavodoxin reductase and flavodoxin system supports catalysis by heterologous P450 enzymes, such as CYP17, with an FMN semiquinone of flavodoxin serving as the electron donor [8]. These studies collectively indicate that deriving a catalytic mechanism based on equilibrium redox potentials alone may not be reliable.

In this paper we studied the mechanism of house fly P450 reductase and addressed the following aspects of catalysis: (a) stoichiometry of cytochrome *c* reduction by NADPH in the presence of P450 reductase excess; (b) role of FMN semiquinone in reduction of cytochrome *c*.

2. Materials and methods

Expression in *E. coli*, purification and quantitation of P450 reductase were carried out as described [9,10]. Cytochrome *c* reductase activity was measured as detailed earlier [10].

2.1. Synthesis of [^{32}P]NADP(H) and interaction with P450 reductase

The reaction mixture (100 μl) contained 2.5 mM NAD, 200 μM ATP, 5 mM $MgCl_2$, 0.5 mCi [γ-^{32}P]ATP, and 0.3 U/ml NAD kinase in 100 mM Tris-HCl pH 7.7. After 4 h incubation at 25°C, [^{32}P]NADP was purified by HPLC on a Vydac 301TP104 column (4.6×250 mm) developed with a linear gradient of 0.05–0.4 M K-acetate buffer pH 5.0 over 60 min at a flow rate of 1 ml/min. [^{32}P]NADPH was synthesized by incubation of [^{32}P]NADP with glucose 6-phosphate and 0.2 U/ml glucose 6-phosphate dehydrogenase for 1 h at 25°C. Glucose 6-phosphate dehydrogenase was removed by ultrafiltration on a 30K Centricon microconcentrator. [^{32}P]NADP and [^{32}P]NADPH comigrated with authentic standards during TLC on PEI-cellulose plates developed in freshly prepared 0.5 M NH_4HCO_3. R_f values for NADPH and NADP were 0.41 and 0.62, respectively. Specific radioactivity of [^{32}P]NADP(H) was 660 cpm/pmol.

Oxidation of [^{32}P]NADPH by P450 reductase was measured by

*Corresponding author. Fax: (1) (520) 626-8058.
E-mail: marat@ag.arizona.edu

Abbreviations: CYP6A1, cytochrome P450 6A1; P450 reductase, NADPH-cytochrome P450 reductase; P450BM3, cytochrome $P450_{BM3}$, isolated from *Bacillus megaterium*; PEI-cellulose, polyethyleneimine-impregnated cellulose

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mixing 5 μl of 4 μM enzyme with 5 μl of 21.6 μM nucleotide. The reaction was stopped by addition of 6 μl of 10% SDS solution and 1.5 μl was applied on a PEI-cellulose plate. After developing the plates as described above, the spots corresponding to [^{32}P]NADP and [^{32}P]NADPH were cut out, placed in scintillation vials containing 5 ml of 1 M KH_2PO_4 and radioactivity counted by Cerenkov irradiation.

2.2. Stopped flow experiments

The experiments were carried out as described [9]. For cytochrome *c* reduction assays one syringe contained 2.3 μM P450 reductase and the second syringe contained 10 μM cytochrome *c* and 1.2 μM NADPH. Equal volumes of the solutions were mixed at 25°C and the absorbance increase was recorded at 550 nm. The reactions followed first order kinetics and rate constants were obtained by a best fit to a single exponential function. Stoichiometry of cytochrome *c*/NADPH was calculated as a ratio of the amount of cytochrome *c* reduced ($\varepsilon_{550} = 21$ mM^{-1} cm^{-1}) to the amount of NADPH added ($\varepsilon_{340} = 6.2$ mM^{-1} cm^{-1}).

3. Results

3.1. NADPH oxidation by the house fly P450 reductase

The house fly P450 reductase is isolated as a stable fully oxidized flavoprotein [10]. To determine whether house fly P450 reductase requires a priming reaction, we measured a stoichiometry of NADPH oxidation. If a priming reaction does not occur, the enzyme will rapidly oxidize 1 mol of NADPH. In contrast, if a priming reaction is required, the enzyme will rapidly oxidize more than 1 mol of NADPH. The fully oxidized enzyme was incubated with an excess of [^{32}P]NADPH, and the amount of oxidized nucleotide was determined. The results of this experiment (Fig. 1) demonstrate that house fly P450 reductase can rapidly (within the first time point of 3 s) oxidize only 1 mol of [^{32}P]NADPH. Stopped flow measurements of FAD reduction gave a rate constant of 26 s^{-1} [10]. A second mol of [^{32}P]NADPH is oxidized in about 1 min ($k \approx 0.01$ s^{-1}), too slow to be a part of the catalytic cycle. Absorption and EPR spectroscopy confirmed that P450 reductase used in these experiments was fully oxidized [10]. Therefore, house fly P450 reductase can react with NADPH rapidly only when the flavoprotein is fully oxidized.

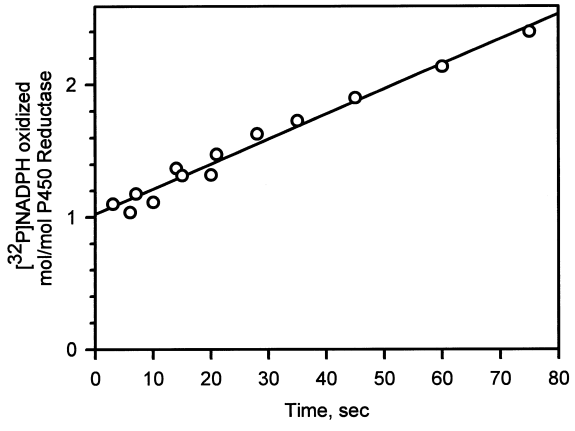

Fig. 1. [^{32}P]NADPH oxidation by house fly P450 reductase. The enzyme (2 μM) was incubated with 10.8 μM [^{32}P]NADPH for the times indicated. The amount of NADPH oxidized was determined after nucleotide separation by TLC on PEI-cellulose. The line is the linear regression through the experimental points.

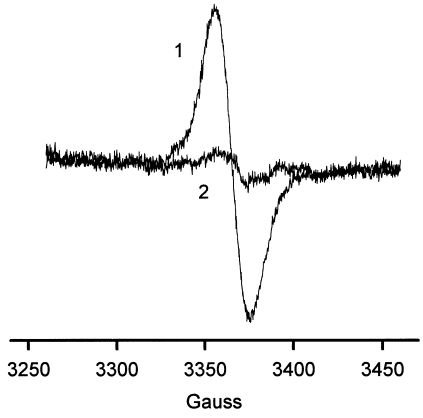

Fig. 2. EPR spectra of the P450 reductase reduced by substoichiometric NADPH in the presence or absence of cytochrome *c*. P450 reductase (40 μM) solution containing 0.25% Emulgen 911 was mixed under aerobic conditions with an equal volume of 32 μM NADPH, and samples were incubated for 1 h on ice with no cytochrome *c* added (spectrum 1), or for 10–15 s with 50 μM cytochrome *c* present (spectrum 2). The spin concentration in the samples corresponded to 0.54 and 0.025 mol/mol of the P450 reductase.

3.2. Cytochrome c reduction at [NADPH] < [P450 reductase]

NADPH delivers two reducing equivalents to the FAD site of P450 reductase in the form of a hydride ion. On the other hand, cytochrome *c* is a one-electron acceptor at the FMN site [11–13]. We measured the rate and stoichiometry of cytochrome *c* reduction at [NADPH] < [P450 reductase] to determine if FMN semiquinone can serve as catalytic intermediate. The addition of 0.6 μM NADPH to 1.15 μM P450 reductase resulted in an essentially stoichiometric transfer of two reducing equivalents to cytochrome *c* in a monophasic first order reaction with a rate constant of 14 s^{-1} (Table 1, exp. 1). The reduction of two equivalents of cytochrome *c* with first order kinetics ($k = 14$ s^{-1}) indicates that the second mol of cytochrome *c* is reduced either with the same rate or faster than the first one, because house fly P450 reductase has only one cytochrome *c* binding site as shown by its Michaelis-Menten kinetics [10]. Results similar to those presented in Table 1 were obtained with 1–5 μM P450 reductase and with 0.12–0.70 mol of NADPH added per mol of enzyme (data not shown).

Under conditions of P450 reductase excess (Table 1, exp. 1), the pseudomonomolecular rate constant of NADPH binding by the enzyme is [P450 reductase] $\times k_1 = 60.4$ s^{-1} which is faster than the rate of cytochrome *c* reduction of 14.2 s^{-1} (Table 1, exp. 1), while hydride ion transfer occurs with a rate constant of 26 s^{-1} (rate constants are from [10]). These calculations demonstrate that almost all NADPH is bound and oxidized before significant cytochrome *c* reduction occurs. Additionally, in the presence of nucleotide excess, house fly P450 reductase can rapidly oxidize only 1 mol of NADPH (Fig. 1). Thus the reaction measured (Table 1, exp. 1) reflects the single turnover reduction of cytochrome *c* after P450 reductase interaction with only 1 mol of NADPH.

To confirm that stoichiometric transfer of electrons to cytochrome *c* at [NADPH] < [P450 reductase] occurs, we used EPR to measure flavin semiquinone formation. P450 reductase (40 μM) was mixed with an equal volume of 32 μM NADPH in the presence of cytochrome *c*, and the sample was frozen in liquid nitrogen 10–15 s after mixing and EPR

Table 1
Reactivity of P450 reductase:NADPH complex and 'air-stable' semiquinone form towards cytochrome *c*

	Reaction conditions	Cytochrome *c*/NADPH (mol/mol)	Reaction rate (s^{-1})
1	1.15 μM reductase, 0.6 μM NADPH	1.75 ± 0.11 (9)	14.2 ± 2.2 (6)[a]
2	0.0025 μM reductase, 0.6 μM NADPH	1.84 ± 0.03 (6)	14.4 ± 1.1 (6)[b]
3	'Air-stable' semiquinone	N.D.[c]	0.005 ± 0.001 (3)

Cytochrome *c* reduction was measured in the presence of 5 μM cytochrome *c*. Stopped flow kinetic measurements were carried out at 1.15 μM P450 reductase and 0.6 μM NADPH. Steady-state cytochrome *c* reduction was measured in the presence of 0.6 μM NADPH and 0.0025 μM house fly P450 reductase. The 'air-stable' semiquinone form was prepared by incubation of 1.5 μM house fly P450 reductase with 1.5 μM NADPH for 10 min. Cytochrome *c* was then added and the reaction was followed at 550 nm. Values are means ± S.D. of (*n*) experiments.
[a]Measured by stopped flow kinetics.
[b]Steady-state cytochrome *c* reduction.
[c]N.D., not determined.

spectra were recorded (Fig. 2). Very little flavin semiquinone could be detected, demonstrating that at [NADPH] < [P450 reductase] a complete transfer of reducing equivalents to cytochrome *c* does indeed occur and the fully oxidized flavoprotein is regenerated. Because NADPH is a two-electron donor, while cytochrome *c* is a one-electron acceptor, rapid stoichiometric reduction of cytochrome *c* at [NADPH] < [P450 reductase] indicates that at least one reducing equivalent is transferred from a one-electron reduced enzyme, that is an FMN semiquinone form, which must therefore be a catalytic intermediate.

The reaction at [NADPH] < [P450 reductase] was compared to that in the presence of nucleotide excess. The results (Table 1, exp. 2) show that both the rate and stoichiometry of cytochrome *c* reduction in the presence of NADPH excess are close to those measured at [NADPH] < [P450 reductase]. Thus rapid and stoichiometric cytochrome *c* reduction at [NADPH] < [P450 reductase] represents normal catalytic turnover.

3.3. Reactivity of the air-stable semiquinone

House fly P450 reductase can readily form an 'air-stable' semiquinone form in the presence of stoichiometric concentrations of NADPH [10]. EPR measurements confirmed that the enzyme incubated with 0.8 equivalents of NADPH for 1 h contained 0.54 mol of free radical per mol of enzyme (Fig. 2) as expected from its slow oxidation [10].

To evaluate the catalytic competence of this FMN semiquinone form of the P450 reductase, the enzyme was first incubated with equimolar amounts of NADPH for 10 min, then cytochrome *c* was added, and its reduction was followed by absorbance increase at 550 nm. The results (Table 1, exp. 3) showed that the 'air-stable' semiquinone form of the house fly P450 reductase catalyzed reduction of cytochrome *c* with a first order rate constant of 0.005 s^{-1}, or almost 3000 times slower than the rate of cytochrome *c* reduction by the catalytic intermediate.

4. Discussion

Our results demonstrate that house fly P450 reductase can rapidly oxidize only 1 mol of [^{32}P]NADPH, while oxidation of a second nucleotide by a partially reduced house fly P450 reductase is too slow to account for the fast rate of catalysis (Fig. 1). Moreover, reduction of 2 mol of cytochrome *c* for each mol of NADPH with a catalytically competent rate occurs even when the nucleotide concentration is below that of the enzyme (Table 1). Thus, no priming reaction is needed for the house fly microsomal P450 reductase to reduce cytochrome *c* with high rates, and an FMN semiquinone of the one-electron reduced enzyme rapidly reduces cytochrome *c*, completing the catalytic turnover and regenerating the fully oxidized P450 reductase. FMN semiquinone also reduces cytochrome *c* with catalytically competent rate in P450BM3 [5] and sulfite reductase [6]. In addition, an FMN semiquinone reduces the heme domain in fusion proteins, such as P450BM3 and nitric oxide synthase [5,7].

Neither two- nor one-electron reduced P450 reductase is able to oxidize a second mol of NADPH with a rate sufficient to account for the catalytic turnover of a physiological acceptor such as CYP6A1. Indeed, the second NADPH is oxidized with a rate constant of about 0.01 s^{-1} (Fig. 1), which is two orders of magnitude slower than reduction of CYP6A1 and b_5, or CYP6A1 catalytic turnover in the presence of b_5 [9]. We conclude therefore that the catalytic mechanism of house fly P450 reductase with physiological electron acceptors is similar to that described for the flavoprotein domain of P450BM3 [5], and can be summarized as follows. NADPH binds to the fully oxidized enzyme, and rapid hydride ion transfer produces a two-electron reduced flavoprotein with two electrons located on different flavin cofactors. Both electrons are transferred to an electron acceptor from an FMN semiquinone and the reduction state of house fly P450 reductase cycles in a 0-2-1-0 sequence.

In contrast, P450 reductases from mammalian microsomes were proposed to cycle between either 1-3 or 2-4 reduction states (see [2] for review), implying that P450 reductase must not oxidize below the one-electron reduced state. However, a number of early observations disagree with either of the mechanisms. (i) EPR measurements showed that P450 reductase of the isolated liver microsomes was fully oxidized, and flavin semiquinone is formed upon addition of NADPH [14]. (ii) In early measurements of the stoichiometry of cytochrome *c* reduction [15] it was found that at least two thirds of the reducing equivalents were transferred from NADPH to cytochrome *c* with high rates under single turnover conditions, suggesting heterogeneity of the FMN semiquinone enzyme form, a fraction of which is catalytically competent. (iii) As shown by flash photolysis [16], electron transfer from FAD semiquinone to the fully oxidized FMN occurs with a rate constant of 70 s^{-1}, close to the measured rates of hydride ion transfer of 28 s^{-1} [17] and steady-state cytochrome *c* reduction of 85 s^{-1} [18]. However, a second electron is transferred to the FMN semiquinone with a rate of only 15 s^{-1}, i.e. 5–6 times slower than cytochrome *c* is reduced under steady-state conditions, making FMN hydroquinone an unlikely catalytic intermediate. Stopped flow measurements also demonstrated that formation of the FMN hydroquinone occurs with

the rate constant of only 5.8 s^{-1} in the presence of NADPH [17], too slow to account for fast cytochrome *c* reduction. These facts taken together suggest that the catalytic role of FMN semiquinone may have been overlooked in the rat liver P450 reductase perhaps because of the formation of a catalytically inactive 'air-stable' semiquinone form that is different from the catalytic intermediate.

Our results demonstrate that the FMN semiquinone form of the house fly P450 reductase can exist in two different conformations. One is the catalytic intermediate. Another one is the so called air-stable semiquinone form which reduces cytochrome *c* 10^3 times more slowly (Table 1), and is clearly not an intermediate of catalysis, but rather represents a sluggish enzyme form analogous to the inactive three-electron reduced flavoprotein of P450BM3 [5,19]. This 'air-stable' semiquinone form of the enzyme is unable to rapidly reduce cytochrome *c* despite the fact that the reduction is thermodynamically favored by +350 mV. This suggests that the 'air-stable' semiquinone enzyme is kinetically stabilized due to an inactive conformation of the protein. We demonstrated in our previous studies that NADP(H) binding is critical for maintaining FAD and FMN electron transfer properties in P450BM3 [5] and house fly P450 reductase [10]. A number of recent observations from other laboratories support our hypothesis on the role of nucleotide binding in diflavin reductase catalysis [20,21]. We suggest that formation of the inactive 'air-stable' semiquinone is caused by a slow (compared to the catalytic turnover) conformational change altering enzyme interaction with the bound nucleotide. Understanding the mechanism by which nucleotide binding controls electron transfer properties of FAD and FMN will be essential for our understanding of electron transfer by flavoproteins.

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