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Reduction of cytochrome b_5 by NADPH–cytochrome P450 reductase

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Abstract

The reduction of mammalian cytochrome b_5 (b_5) by NADPH–cytochrome P450 (P450) reductase is involved in a number of biological reactions. The kinetics of the process have received limited consideration previously, and a combination of pre-steady-state (stopped-flow) and steady-state approaches was used to investigate the mechanism of b_5 reduction. In the absence of detergent or lipid, a reductase–b_5 complex is formed and rearranges slowly to an active form. Electron transfer to b_5 is rapid within this complex (>30 s^{-1} at 23 °C), as fast as to cytochrome *c*. With excess b_5 present, a burst of reduction is observed, consistent with rapid electron transfer to one or two b_5 molecules per reductase, followed by a subsequent rate-limiting event. In detergent vesicles, the reductase and b_5 interact rapidly but electron transfer is slower (~3 s^{-1} at 23 °C). Experiments with dimyristyl lecithin vesicles yielded results intermediate between the non-vesicle and detergent systems. These steady-state and pre-steady-state kinetics provide views of the different natures of the reduction of b_5 by the reductase in the absence and presence of vesicles. Without vesicles, the encounter of the reductase and b_5 is rapid, followed by a slow reorganization of the initial complex (~0.07 s^{-1}), very fast reduction, and dissociation. In vesicles, encounter is rapid and the slow step (~3 s^{-1}) is reduction within a complex less favorable for reduction than in the non-vesicle systems.
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Keywords: Cytochrome b_5; NADPH–cytochrome P450 reductase; Reduction of cytochrome b_5; Pre-steady-state kinetics; Electron transfer; Membrane proteins; Phospholipid vesicles; Non-ionic detergents; Cytochrome *c*; Reduction of cytochrome *c*; Detergent vesicles; Tergitol NP-10; Dimyristoyl lecithin; Ionic strength

Three major proteins involved in microsomal electron transfer reactions are P450,[1] NPR, and b_5. These proteins are involved in a wide variety of oxidations and reductions of various endobiotic and xenobiotic chemicals [2–6]. b_5 is a component of the NADH-dependent fatty acid desaturase [7,8] and elongation [9,10] pathways. In addition, b_5 is also involved in the reduction of hemoglobin [11], the synthesis of plasmalogens [6,12], and the synthesis of methionine [13] and can enhance the activities of many P450 reactions [6,14]. b_5 is reduced by an NADH-dependent flavoprotein, NADH-b_5 reductase [15,16]. However, b_5 is also known to be reduced by NPR [17,18].

The catalytic mechanism of NPR is complex, due in large part to the presence of two flavins [5]. Most of the mechanistic studies have been done either with NPR alone or with artificial electron acceptors, for example $Fe(CN)_6^{3-}$, cytochrome *c* [5,19]. Less mechanistic work has been done with the natural electron acceptors b_5 and P450s due to the inherent instability of the reduced products in the presence of O_2. However, the NPR-mediated reduction of many P450s has been studied [20–22]. The rates of reduction of the ferric enzymes are usually rapid; the rates of electron transfer to ferrous–oxygen complexes are not well-characterized [23,24].

Some work on the kinetics of b_5 reduction has been reported. Enoch and Strittmatter [17] reported an

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[1] *Abbreviations:* b_5, cytochrome b_5; P450, cytochrome P450 (also termed "heme–thiolate protein P450" [1]); NPR, NADPH-P450 reductase; di-14:0 GPC, L-α-dimyristoyl-*sn*-glycero-3-phosphocholine; di-12:0 GPC, L-α-dilauroyl-*sn*-glycero-3-phosphocholine.

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apparent first-order reduction rate of b_5 by NPR of 1.1 s^{-1} in rabbit liver microsomes. Purified beef liver NPR reduced beef liver b_5 at a first-order rate of 1.7 s^{-1} (30 °C) in di-14:0 GPC vesicles [17]. A rate of 25 s^{-1} was reported in detergent vesicles. Wu et al. [25] examined several site-directed mutants of recombinant rat b_5 by NPR. When the two proteins were mixed together from individual syringes, very slow reduction was observed. When the two proteins were mixed in a typical di-12:0 GPC system used in reactions, triphasic reduction was observed with only 1/6 of the b_5 reduced in the fast phase (with a rate of 4 s^{-1}). With detergent vesicles, mixing NPR and b_5 from separate syringes yielded reduction rates identical with those obtained with a premixed solution of the two proteins ($k = 0.9$ s^{-1}) [25]. Some work was done in this laboratory, particularly in terms of b_5 interactions with P450 reduction by NPR [22]. Another interesting point is that studies using plasmon resonance [26] and ELISA assays [27] have failed to detect stable complexes of NPR and b_5.

A series of measurements of rates of the b_5 reduction were done to clarify the kinetics of the electron transfer pathway with the NPR and b_5 in the absence and presence of detergent and phospholipid vesicles. Both steady-state and pre-steady-state (stopped-flow) experiments were done, and the collective results were used to interpret the kinetics of the reduction process. The rate-limiting step differs between systems using only the purified proteins and vesicle-based system.

Materials and methods

Enzymes

Recombinant (rat) NPR was expressed in *Escherichia coli* and purified as described [28]. Horse heart cytochrome *c* was purchased from Sigma Chemical (St. Louis, MO) and used without further purification. Recombinant (human) b_5 was expressed in *E. coli* JM109 cells from a plasmid [pSE420 (Amp)] kindly provided by Satoru Asaki (Takeda Pharmaceutical, Osaka, Japan). The protein was solubilized and purified to electrophoretic homogeneity using modifications of the DEAE–cellulose and other chromatography methods described elsewhere [29,30]. Apo-b_5 was prepared by removal of heme from b_5 [31,32].

Vesicles

Di-14:0 vesicles were prepared using sonication (3×10 min at a 4 W power setting with a VWR Branson Model 450 sonicator) (VWR International, Marietta, GA) and ultracentrifugation (30 min at $10^5 g$) (all at 23 °C) as described by Strittmatter et al. [33]. The residues were stored at room temperature after preparation and used within 2 days. Tergitol NP-10 (from Sigma) was dissolved in H_2O to prepare a 2.5% stock solution (w/v), which was diluted for use.

Spectroscopy

Concentrations of b_5 [34] and NPR [35] were measured using UV–visible spectroscopy. Steady-state measurements of rates of NPR reduction were made in 0.3 M potassium phosphate buffer (pH 7.7) at 23 °C with 2.0 nM concentrations of NPR, using an OLIS/Cary 14 instrument (On-Line Instrument Systems, Bogart, GA). Rates of cytochrome *c* reduction were measured (aerobically) with 40 μM cytochrome *c*, using $\Delta\varepsilon_{550} = 21{,}000$ M^{-1} cm^{-1} [36]. Rates of b_5 reduction were determined in anaerobic glass cuvettes [32,37,38], using $\Delta\varepsilon_{424} = 100{,}000$ M^{-1} cm^{-1} [39,40].

Stopped-flow measurements were made (aerobically) using an OLIS RSM-1000 instrument in the rapid-scanning mode. All rates were measured at 23 °C. In general, experiments of $\leqslant 2$ s involved collection of 1000 scans s^{-1}. In longer runs, a signal averaging (usually 62 scans s^{-1}) mode was employed. Data were fit to equations using the fitting programs provided with the instrument. The parameters reported here are generally representative of or are means of $\geqslant 3$ experiments. The reduction of b_5 yields an increase in A_{424} and decrease in A_{409}. Both changes were usually recorded, and in most cases the kinetic fits were similar. In some cases the increase in A_{559} associated with reduction was also used. The reduction of the flavins of NPR produces a small decrease in A_{424} (not an increase, as reported by Wu et al. [25]), but the $\Delta\varepsilon$ associated with this is <5000 M^{-1} cm^{-1} and does not interfere with the changes in the b_5 spectra (vide infra) in the experiments done here, because of the difference in extinction coefficients.

Results

Reduction of NPR and cytochrome c

The rate of reduction of NPR measured following mixing with a saturating concentration of NADPH (150 μM) was 33 s^{-1}, as measured by the rate of decrease of the absorbance at 455 or 380 nm, in agreement with previous studies [41]. The steady-state rate of cytochrome *c* reduction (measured with 40 μM cytochrome *c* and 2 nM NPR) was 77 s^{-1} at 23 °C in 0.3 M potassium phosphate buffer (pH 7.7), corresponding to a specific activity of 59,000 nmol cytochrome *c* reduced min^{-1} $(mg\ protein)^{-1}$ which is in good agreement with literature values [5,17]. The discrepancy (33 s^{-1} vs. 77 s^{-1}) may be due to faster reduction of the flavin pair at an intermediate redox stage (in steady-state catalysis [5])

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