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Short Communication

Cytochrome P450 2S1 is Reduced by NADPH-Cytochrome P450 Reductase

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ABSTRACT:

Cytochrome P450 (P450) 2S1 is one of the orphan P450s without a clear physiological function. Controversy has arisen as to whether it can interact with NADPH-P450 reductase and accept electrons. The reduction of 1,4-bis{[2-(dimethylamino-*N*-oxide)ethyl]amino}-5,8-dihydroxyanthracene-9,10-dione (AQ4N) by P450 2S1 was confirmed, and the NADPH consumption rates were measured aerobically and anaerobically in the absence and presence of the drug. The reduction kinetics of P450 2S1 were rapid, as measured by stopped-flow kinetics. These results confirm that P450 2S1 can be reduced by NADPH-P450 reductase and suggest normal mixed-function oxidase roles of P450 2S1 to be revealed.

Introduction

Cytochrome P450 2S1 is one of the orphan P450s with unknown physiological function (Guengerich et al., 2010, 2011). P450 2S1 mRNA can be detected in human skin and liver, and there are also reports of expression in trachea, lung, stomach, small intestine, and spleen (Guengerich et al., 2011). The gene is regulated by the *Ah* receptor (Rivera et al., 2002, 2007). Although P450 2S1 has been expressed in *Escherichia coli*, purified, and studied, its substrate specificity is still controversial (Wu et al., 2006; Guengerich et al., 2011). To explain the limited catalytic activity of P450 2S1, Bui and Hankinson (2009) attempted to measure the formation of the Fe^{2+}·CO complex under aerobic conditions and concluded that P450 2S1 is not capable of interacting with NADPH-P450 reductase nor of accepting electrons from the reductase (Bui et al., 2009, 2011). In contrast, Nishida et al. (2010) recently observed that a Fe^{2+}·CO complex formed anaerobically. In that study, P450 2S1 was reported to reduce the prodrug 1,4-*bis*{[2-(dimethylamino-*N*-oxide)ethyl]-amino}-5,8-dihydroxyanthracene-9,10-dione (AQ4N) to its mono *N*-oxide intermediate 1-{[2-(dimethylamino-*N*-oxide)ethyl]-amino}-4-{[2-(dimethylamino)ethyl]amino}-5,8-dihydroxyanthracene-9,10-dione (AQ4M) and finally to 1,4-*bis*{[2-(dimethylamino)ethyl]amino}-5,8-dihydroxyanthracene-9,10-dione (AQ4). A net 2-electron transfer mechanism was proposed (for AQ4M formation) (Nishida et al., 2010). In this study, we reproduced the anaerobic reduction of AQ4N, measured NADPH oxidation rates, and measured the reduction kinetics using a stopped-flow apparatus. Our results confirm that P450 2S1 can efficiently accept electrons from NADPH-P450 reductase.

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Materials and Methods

Chemical and Reagents. Desferoxamine, mitoxantrone, protocatechuate, and protocatechuate dioxygenase were purchased from Sigma-Aldrich (St. Louis, MO). AQ4N, AQ4M, and AQN were gifts from Dr. Klaus Pors (University of Bradford, West Yorkshire, United Kingdom). Human P450 2S1 (Wu et al., 2006) and rat NADPH-P450 reductase (Hanna et al., 1998) were expressed in *E. coli* and purified as reported previously.

Anaerobic Reduction. Anerobic reduction experiments were performed using an OLIS RSM-1000 stopped-flow instrument (On-Line Instrument Systems, Bogart, GA). Samples (in glass tonometers) were deaerated using an argon/vacuum manifold as described previously (Guengerich et al., 2004), using a protocatechuate/protocatechuate dioxygenase oxygen-scrubbing system (Patil and Ballou, 2000). P450 2S1 (2 μM) was preincubated with NADPH-P450 reductase (4 μM), 120 μM L-α-1,2-dilauoryl-*sn*-glycero-3-phosphocholine, 100 mM potassium phosphate buffer (pH 7.4), and AQ4N (200 μM) and reduced upon the addition of NADPH (150 μM) from a second syringe. Rates were analyzed using the OLIS software and in GraphPad Prism (GraphPad Software Inc., San Diego, CA).

Enzyme Activity of P450 2S1 toward AQ4N. Reactions were carried out in duplicate in 1.5-ml Eppendorf tubes at 37°C. Anerobic incubations were carried out under a nitrogen atmosphere inside of a glovebox (Labconco Protector Controlled Atmosphere). Enzyme reaction mixtures typically contained 0.1 μM P450 2S1, 0.2 μM NADPH-P450 reductase, 160 μM L-α-1,2-dilauoryl-*sn*-glycero-3-phosphocholine, 1 mM desferoxamine, 100 mM potassium phosphate buffer (pH 7.4), and 200 μM AQ4N (Nishida et al., 2010). After preincubation for 5 min, the reactions were started by the addition of an NADPH-generating system (Guengerich and Bartleson, 2007), and aliquots were terminated by the addition of three volumes of CH_3OH (with 5 μM mitoxantrone as an internal standard) at 0, 1, 2, 3, 4, 5, and 6 min. Samples were prepared and analyzed by high-performance liquid chromatography, as described previously (Swaine et al., 2000), with a Thermo Hypersil GOLD octadecylsilane column (150 × 2.1 mm i.d.; Thermo Fisher Scientific, Waltham, MA) using isocratic elution with a 50 mM NH_4HCO_2 buffer (pH 3.6)/CH_3CN mixture (89:11, v/v). Formation of AQ4M was used to measure enzyme activity.

ABBREVIATIONS: P450, cytochrome P450; AQ4N, 1,4-*bis*{[2-(dimethylamino-*N*-oxide)ethyl]amino}-5,8-dihydroxyanthracene-9,10-dione; AQ4M, 1-{[2-(dimethylamino-*N*-oxide)ethyl]amino}-4-{[2-(dimethylamino)ethyl]amino}-5,8-dihydroxyanthracene-9,10-dione; AQ4, 1,4-*bis*{[2-(dimethylamino)ethyl]amino}-5,8-dihydroxyanthracene-9,10-dione.

TABLE 1

NADPH oxidation rates

		v (min^{-1}) [a]
Plus air	Minus AQ4N	23.3 ± 2.6
	Plus AQ4N	45.7 ± 4.7
Minus air	Minus AQ4N	12.7 ± 2.9
	Plus AQ4N	28.2 ± 0.8

[a] Results are means of duplicate experiments ± range.

Determination of NADPH Consumption Rates. NADPH oxidation rates for P450 2S1 were determined using 0.05 μM P450 2S1, 0.10 μM NADPH-P450 reductase, 160 μM L-α-1,2-dilauoryl-*sn*-glycero-3-phosphocholine, 1 mM desferoxamine, and 100 mM potassium phosphate buffer, pH 7.4. For the determination of NADPH consumption rates under anaerobic conditions, reconstituted enzyme mixtures were deaerated in all-glass anaerobic cuvettes using an argon/vacuum manifold as described previously (Guengerich et al., 2004). Reconstituted enzymes were preincubated for 5 min at 37°C in the presence or absence of AQ4N (200 μM). Reactions were initiated with the addition of NADPH to a final concentration of 150 μM, and A_{340} was monitored (Cary 14/OLIS instrument; On-Line Instrument Systems). Rates were calculated using the value $\Delta\varepsilon_{340} = 6.22\ mM^{-1}\ cm^{-1}$. Experiments were conducted in duplicate.

Results and Discussion

The reduction of AQ4N to AQ4M is proposed to be a net 2-electron transfer mechanism (Nishida et al., 2010). We reproduced the hypoxic activation of AQ4N by P450 2S1, measured to be 18.2 min^{-1} under anaerobic conditions and $<0.5\ min^{-1}$ under aerobic conditions.

The steady-state rates of oxidation of NADPH by P450 2S1 in the presence of NADPH-P450 reductase were also measured (Table 1). The higher oxidation rate under aerobic conditions confirms the acceptance of electrons by P450 2S1 from NADPH-P450 reductase. Because the rate of AQ4N catalysis under aerobic conditions is very low, the increased NADPH consumption in the presence of AQ4N presumably comes from electron transfer to form partially reduced oxygen products instead of being transferred to AQ4N.

The rate of product formation was measured to be 18.2 min^{-1}. The NADPH consumption rate without air in the presence of AQ4N was 28.2 min^{-1} and 12.6 min^{-1} in the absence of AQ4N, suggesting roughly quantitative coupling efficiency, i.e., all additional electrons are transferred to substrate AQ4N.[1]

The reduction step can be studied by monitoring the formation of the Fe^{2+}·CO complex near 450 nm in the absence of O_2, which will compete with CO and lead to P450 reoxidation (Gigon et al., 1969; Guengerich and Johnson, 1997). Our kinetic reduction data, measured in a stopped-flow apparatus (Fig. 1), clearly shows a rapid increase in A_{450}, indicating the acceptance of electrons by P450 2S1 from NADPH-P450 reductase.

Bui and Hankinson (2009) reported that P450 2S1 could not be reduced by NADPH-P450 reductase and that several catalytic activities of P450 2S1 could be observed if reactions were supported by oxygen surrogates, e.g., alkyl hydroperoxides (Bui et al., 2009, 2011). In contrast, Nishida et al. (2010) reported the observation of a Fe^{2+}·CO complex produced by P450 2S1 and AQ4N (rate not measured). A serious caveat in the work of Bui and Hankinson (2009) is that the reduction work was done aerobically, conditions under which ferrous P450s are rapidly reoxidized (Guengerich et al., 1976). Nishida et al. (2010) also pointed out that the conclusion of Bui and collegues (Bui and Hankinson, 2009; Bui et al., 2009, 2011) that P450 2S1 normally uses lipid peroxides for its catalytic function is invalid, in that many P450s can react with lipid hydroperoxides through a shunt pathway that generates lipid alkoxy and peroxy radicals. These radicals can enter co-oxidation reactions outside of P450 active sites (Mansuy et al., 1982; Ortiz de Montellano, 1995). In another recent publication, Bui et al. (2011) reported isomerization activity of P450 2S1 in a NADPH-independent manner. However, such isomerization activity has been seen with several other P450 enzymes, and its physiological role is not validated (Weiss et al., 1987; Chang et al., 1996).

In conclusion, our results are in agreement with previous findings (Nishida et al., 2010) that P450 2S1 can accept electrons from NADPH-P450 reductase. A physiological role of P450 2S1, if it exists, remains to be revealed.

[1] The NADPH oxidation rate measured in the absence of air is attributed to trace oxygen in the system, corresponding to ~6 nmol consumed in 3 min (~5 ppm in gas phase).

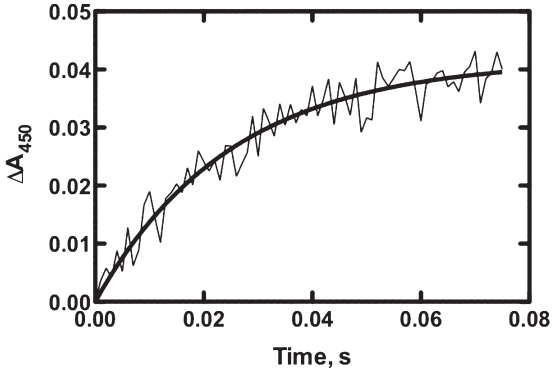

FIG. 1. Reduction kinetics of P450 2S1 (with AQ4N). Stopped-flow absorbance trace of the reduction of P450 2S1 (2 μM) by NADPH-P450 reductase (4 μM) in the presence of L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine (120 μM) and AQ4N (200 μM) upon the addition of NADPH (150 μM). The rate was 39.9 s^{-1} (measured by averaging results from 16 independent shots).

Authorship Contributions

Participated in research design: Xiao, Shinkyo, and Guengerich.

Conducted experiments: Xiao, Shinkyo, and Guengerich.

Performed data analysis: Xiao and Guengerich.

Wrote or contributed to the writing of the manuscript: Xiao and Guengerich.

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