Nishida et al. (2010) recently observed that a Fe$^{2+}$ electrons from the reductase (Bui et al., 2009, 2011). In contrast, capable of interacting with NADPH-P450 reductase nor of accepting complex under aerobic conditions and concluded that P450 2S1 is not.

**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 aeration 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.

**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.

**Enzyme Activity of P450 2S1 toward AQ4N.** Reactions were carried out in duplicate in 1.5-ml Eppendorf tubes at 37°C. An aerobic 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,α-di-lauroyl-sn-glycero-3-phosphocholine, 1 mM desferoxamine, 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.

**Enzyme Activity of P450 2S1 toward AQ4N.** Enzyme activity was measured using an OLIS RSM-1000 stopped-flow instrument (On-Line Instrument Systems, Bogart, GA). Species in (glass tometers) 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 2S1 (4 μM)], 120 μM l-α,α-di-lauroyl-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.

**Materials and Methods.** 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.** An aerobic reduction experiments were performed using an OLIS RSM-1000 stopped-flow instrument (On-Line Instrument Systems, Bogart, GA). Samples (in glass tometers) 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 2S1 (4 μM)], 120 μM l-α,α-di-lauroyl-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.

This work was supported by the National Institutes of Health National Cancer Institute [Grant R37-CA090426 (to F.P.G.); and the National Institutes of Health National Cancer Institute [Grant R37-CA090426 (to F.P.G.)].

Article, publication date, and citation information can be found at http://dmd.aspetjournals.org.

DOI: 10.1124/dmd.111.039321.

**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.

944
Determination of NADPH Consumption Rates. NADPH oxidation for P450 2S1 were determined using 0.05 μM P450 2S1, 0.10 μM NADPH-P450 reductase, 160 μM l-α,α,β,β-4,5-dial-

TABLE 1

<table>
<thead>
<tr>
<th>NADPH oxidation rates</th>
<th>v (min⁻¹) *</th>
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<tbody>
<tr>
<td>Plus air</td>
<td>Minus AQ4N</td>
</tr>
<tr>
<td></td>
<td>Plus AQ4N</td>
</tr>
<tr>
<td>Minus AQ4N</td>
<td>Minus AQ4N</td>
</tr>
<tr>
<td></td>
<td>Plus AQ4N</td>
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* Results are means of duplicate experiments ± range.

The reduction of AQ4N to AQ4M is proposed to be a net 2-electron transfer process. 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.

The reduction step can be studied by monitoring the formation of the Fe²⁺-CO complex near 450 nm in the absence of CO, 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 A₅₅₀, 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 Fe²⁺-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 colleagues (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.

References


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