A novel approach to study the activity and stoichiometry simultaneously for microsomal pentoxyresorufin-*O*-dealkylase reaction

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Abstract A simple approach to study the activity and stoichiometry of cytochrome P-450 IIB1-catalyzed metabolism of pentoxyresorufin (PRF) has been investigated. It involves the continuous spectral analysis of reaction mixture containing PRF, microsomes from phenobarbital-induced rats and NADPH. The kinetics of NADPH consumption, PRF utilization, NADP and resorufin formation was monitored at λ_{max} of 338, 484, 260 and 572 nm, respectively. The stoichiometry of the enzyme reaction tabulated either by specific activity or by V_{max} value showed that 10 molecules of NADPH were required for the conversion of one molecule of PRF to one molecule of resorufin along with 10 molecules of NADP. Further, it was observed that almost six molecules of NADPH are consumed in the incubation mixture devoid of PRF indicating the possibility of metabolism of endogenous substrates. Interestingly, the stoichiometry ratio of 1:1 for PRF and resorufin was established even in the presence of P-450 inhibitors with a lower rate of metabolism. However, the ratio of NADPH to PRF was altered in the presence of inhibitors, suggesting that the simultaneous monitoring of the substrate, electron donor and the products could be useful in understanding the modifications of stoichiometry of electron donor and substrate/product.

Key words: Pentoxyresorufin; Resorufin; Cytochrome P-450 IIB1; Phenobarbital

1. Introduction

Cytochrome P-450 (P-450) is regarded as the collective name of a superfamily of hemoproteins, which carry out the oxidative conversion of various exogenous and endogenous compounds [1–3]. Their principal function requires molecular oxygen, a supply of reducing equivalent from NADPH and a substrate. Spectrophotometry has played a vital role in monitoring oxidation–reduction characteristics and measuring oxidative transformation in a wide variety of substrates by mixed function oxidation reactions [4]. The spectral changes observed during the aerobic steady state of oxidative transformation of many drugs, steroids and polycyclic aromatic hydrocarbons (PAHs) has made difference spectrophotometry an important tool to study various reactions catalyzed by P-450 [5,6].

Various groups of P-450 proteins in a system can easily be modulated by treatment with different drugs, PAHs or other xenobiotics [7–9]. Treatment of rats with phenobarbital (PB) readily induces a P-450 IIB1 family, which exhibits specific dealkylation of pentoxyresorufin (PRF) [10–12]. PRF metabolism is generally monitored using continuous spectrofluorometry, wherein formation of the product resorufin is measured at excitation and emission wavelengths of 522/530 nm and 586 nm, respectively [11,12]. In this communication, a novel spectrophotometric approach to study the activity and stoichiometry simultaneously for the microsomal pentoxyresorufin-*O*-dealkylase (PROD) reaction is presented.

2. Materials and methods

2.1. Chemicals

Dicumarol, metyrapone, NADPH and PRF were purchased from Sigma Chemical Company (St. Louis, MO, USA). SKF 525A was acquired from Smithkline and French Laboratories (Philadelphia, PA, USA), α-Naphthoflavone (α-NF) was a product from Aldrich-Europe (Beerse, Belgium). PB was purchased from Merck and Company (Rahway, NJ, USA). Resorufin was a product of Aldrich Chemical Company (Milwaukee, WI, USA). All other chemicals used were of analytical reagent grade.

2.2. Animal treatment and preparation of microsomes

Druckrey male rats (150 ± 10 g) from the ITRC animal breeding colony were treated with i.p. injections of PB (80 mg/kg body weight) daily for 3 consecutive days in 0.5 ml of saline. The animals were killed 24 h after the last injection. Livers were removed, washed with chilled 0.1 M phosphate buffer pH 7.4 and homogenized. Microsomes and cytosol were prepared as described earlier [13]. Microsomes were resuspended in 0.1 M phosphate buffer pH 7.4 containing 10 mM dithiothreitol, 10 mM EDTA and 20% (v/v) glycerol. The aliquots of microsomal and cytosolic suspensions were frozen at −80°C. Protein content in microsomal and cytosolic fractions was estimated according to the method of Lowry et al. [14].

2.3. PROD protocol

The dealkylation of PRF, NADPH consumed and formation of NADP, resorufin were monitored at 25°C in quartz cuvettes (1 cm path length) using Perkin-Elmer Lambda Bio 20 double beam spectrophotometer. An incubation mixture in a final volume of 3.0 ml contained 50 mM Tris buffer pH 7.5, 25 mM $MgCl_2$, 10 μM PRF and microsomal protein (100–160 μg). The reaction was initiated by the addition of 125 μM NADPH. The reference cuvette contained all the ingredients except PRF and NADPH. Repetitive spectra were recorded every minute from 230 to 650 nm. The rate of formation of resorufin and production of NADP was measured by the increase

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Abbreviations: CO, carbon monoxide; α-NF, α-naphthoflavone; PRF, pentoxyresorufin; PROD, pentoxyresorufin-*O*-dealkylase; P-450, cytochrome P-450; PAHs, polycyclic aromatic hydrocarbons; PB, phenobarbital

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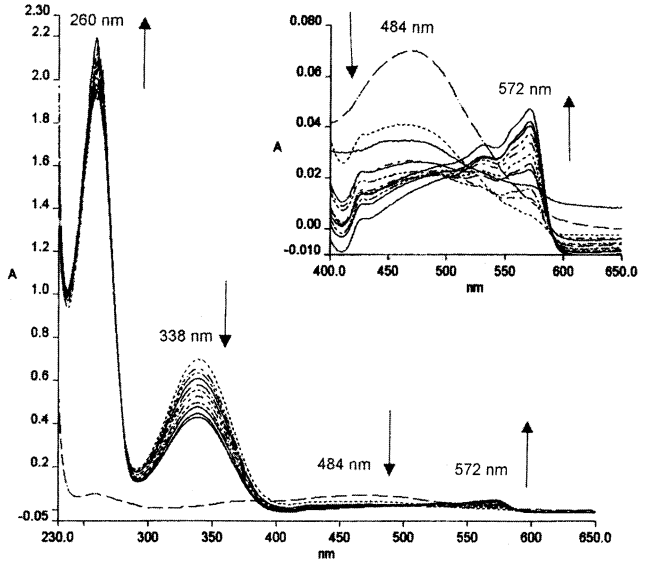

Fig. 1. Time-dependent spectral analysis of PROD reaction mixture. The details of the reaction mixture are described in Section 2 and the reaction was started by the addition of NADPH in the sample cuvette. Repetitive spectra were recorded every min from 230 to 650 nm. The disappearance of NADPH and PRF and the formation of NADP and resorufin were monitored at λ_{max} of 338, 484, 260 and 572 nm respectively. Inset shows the magnified spectra from 400 to 650 nm.

in relative absorbance at 572 nm and 260 nm, respectively. The disappearance of PRF and NADPH during the course of reaction was monitored at λ_{max} of 484 nm and 338 nm, respectively. The calculations of specific activity were made by monitoring the absorbance of standard compounds at respective wavelengths. The extinction coefficients for NADPH and NADP were 6.22 and 18 mM^{-1} cm^{-1}, respectively [15]. The extinction coefficient for resorufin was found to be 73.2 mM^{-1} cm^{-1} [16] while 36.2 mM^{-1} cm^{-1} was calculated for PRF.

2.4. Inhibitor studies

The effect of P-450 inhibitors vis-a-vis metyrapone, SKF-525A, α-NF and the oxidoreductase inhibitor dicumarol on the enzyme activity was measured by preincubation of inhibitors for 1 min prior to addition of substrate and NADPH, in both the cuvettes and repetitive spectra recorded.

The reaction mixture was flushed with carbon monoxide for 1 min to investigate the role of heme protein during enzyme reaction while heat-denatured microsomes were used as negative controls.

3. Results and discussion

Fig. 1 shows the time-dependent spectral characteristics of NADP, NADPH, PRF and resorufin in the reaction mixture. The reaction mixture without NADPH showed a peak of PRF at 484 nm. The 0 min incubation mixture showed respective λ_{max} of NADPH and PRF at 338 and 484 nm, while no peak of the dealkylated product resorufin was evident at 572 nm. However, with a span of 10 min, a time-dependent decrease was observed for NADPH and PRF while an increase in absorbance at 260 nm for NADP was clearly noticed along with formation of resorufin at 572 nm (inset of Fig. 1). It can be deduced from the spectra that NADP production, resorufin formation, NADPH consumption and PRF utilization were consistent throughout the reaction.

Table 1 shows the specific activity of PROD in terms of NADPH and PRF consumed and NADP and resorufin formed. The values of NADPH consumption and PRF utilization were 104.4 and 10.3 nmol/min/mg protein, while the values of resorufin formation and NADP production were 9.6 and 101.1 nmol/min/mg protein (Table 1). The Hanes plots of PRF concentration versus NADP production, resorufin formation, PRF utilization and NADPH consumption are shown in Fig. 2. The V_{max} for NADP and resorufin formation were found to be 67.1 and 6.9 nmol/min/mg protein while those for NADPH and PRF consumption were 69.4 and 6.9 nmol/min/mg protein, respectively. The results of Table 1 and Fig. 2 clearly indicate that there exists a stoichiometry of 10 mol consumption of NADPH for conversion of 1 mol of PRF to 1 mol of resorufin and can be represented as follows:

Table 1
Specific activity of microsomal PROD as a function of PRF utilization, NADPH consumption, NADP production and resorufin formation

PROD (U)	Specific activity
NADP produced (nmol/min/mg protein)	101.1
NADPH consumed (nmol/min/mg protein)	104.4
PRF utilized (nmol/min/mg protein)	10.3
Resorufin formed (nmol/min/mg protein)	9.6

Data from a typical experiment repeated twice with less than 10% variation.

$$\underset{(1)}{PRF} + \underset{(10)}{NADPH} \longrightarrow \underset{(1)}{Resorufin} + \underset{(10)}{NADP^{+}}$$

It was, however, argued that the consumption of NADPH is relatively quite high and may be due to metabolism of endogenous substrates present in the microsomes. Table 2 shows that 69 nmol/min/mg protein of NADPH is consumed in the incubation mixture devoid of PRF. Further, when the reaction mixture was flushed with carbon monoxide prior to NADPH addition, the consumption of NADPH was reduced to 11 nmol/min/mg protein (Table 2). These results suggest that other forms of P-450 in the microsomes may participate in endogenous metabolism of substrates thereby consuming NADPH. Considering endogenous utilization of NADPH in the microsomal system, the actual consumption of NADPH for PRF metabolism could be claimed as 4:1.

To understand whether the activity and stoichiometry of PROD is governed by P-450, or influenced by oxidoreductases, the effect of specific inhibitors was evaluated (Table 3). The specific activity of PROD was found to be substantially inhibited by metyrapone and SKF 525A but not by α-NF, indicating the involvement of P-450 IIB1 enzyme. Fur-

Table 2
Endogenous consumption of NADPH in PB-induced microsomes

Incubation mixture	Specific activity (nmol NADPH consumed/min/mg protein)
PB microsomes+-NADPH−PRF	69.7
PB microsomes+CO+-NADPH−PRF	11.2

Data from a typical experiment repeated twice with less than 10% variation. The reaction mixture in a final volume of 3.0 ml contained 50 mM Tris buffer pH 7.5, 25 mM $MgCl_2$, 100 μg microsomal protein and the reaction was started by the addition of 125 μM NADPH. The reaction was monitored every minute from 230 to 400 nm. In case of inhibition by carbon monoxide, the gas was flushed prior to NADPH addition.

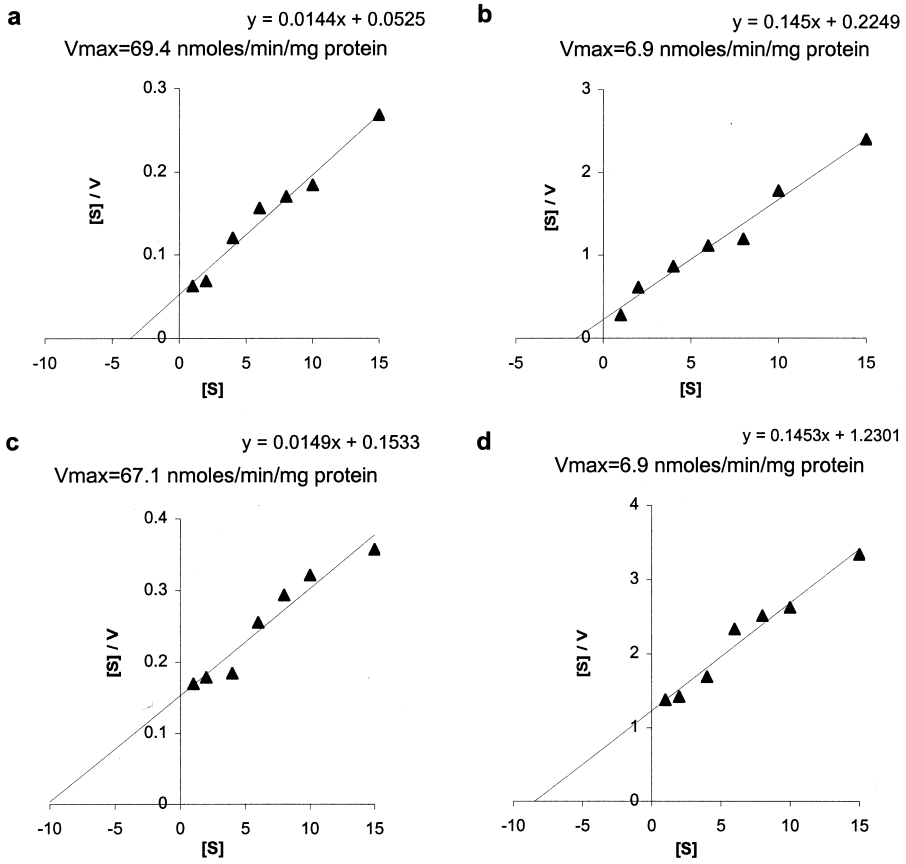

Fig. 2. Hanes plots of PRF concentrations versus (a) NADPH consumption, (b) PRF utilization, (c) NADP production and (d) resorufin formation.

ther, the incubation system flushed with CO also showed an inhibitory effect on the activity of the enzyme suggesting the role of the heme moiety of P-450 in PRF dealkylation. Heat-denatured microsomes showed complete inhibition of PROD activity. Since Nims et al. [17] proposed that NADPH:P-450 reductase could reduce resorufin, it was thought desirable to investigate the role of this protein on the stoichiometry of resorufin production by microsomal PROD. Incubation of dicumarol (10 μM) in the microsomal PROD incubation system did not result in any change in the formation of resorufin and utilization of PRF (Table 3). However, there is a significant decrease in consumption of NADPH or production of NADP (Table 3) which may be due to inhibition of NADPH:P-450 reductase [18]. Thus it can be argued that NADPH:P-450 reductase may have no influence on the stoichiometry of PRF utilization and resorufin formation during the microsomal PROD assay. Furthermore, addition of resorufin in the presence of hepatic cytosol from PB-induced animals and NADPH resulted in reduction of resorufin at 572 nm, while dicumarol completely blocks this reduction process (Fig. 3). This suggests that cytosolic oxidoreductase may change the stoichiometry of resorufin production in the incubation system containing either hepatic S-9 or homogenate fraction.

Table 3
Effect of P-450 and oxidoreductase inhibitors on microsomal PROD activity

Inhibitor	PROD activity			
	NADPH[a]	PRF[b]	NADP[c]	Resorufin[d]
None (control)	96.8	10.2	95.2	9.8
Metyrapone (1 μM)	54.1 (44)	3.0 (71)	49.5 (46)	3.1 (68)
SKF 525A (10 μM)	59.4 (39)	4.2 (59)	57.1 (40)	4.4 (55)
α-NF (10 μM)	86.2 (11)	9.0 (12)	79.7 (16)	8.8 (10)
Dicumarol (10 μM)	64.5 (33)	9.8 (–)	58.9 (38)	9.9 (–)
CO-flushed	11.0 (89)	0.4 (96)	10.1 (89)	0.3 (97)
Heat-denatured	2.3 (98)	0.2 (98)	2.2 (98)	0.1 (99)

Data from a typical experiment repeated twice with less than 10% variation. Specific activity was calculated after recording the spectra after 5 min incubation. Values in parentheses indicate % inhibition when compared to controls.
[a]NADPH consumed (nmol/min/mg protein).
[b]PRF utilized (nmol/min/mg protein).
[c]NADP produced (nmol/min/mg protein).
[d]Resorufin formed (nmol/min/mg protein).

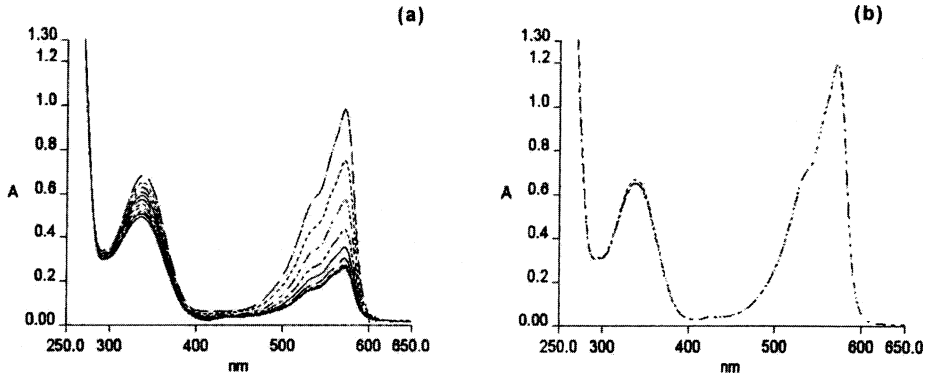

Fig. 3. Time-dependent spectral analysis of cytosolic resorufin quinone reductase activity in the absence and presence of dicumarol. The reaction mixture in a final volume of 3.0 ml contained 50 mM Tris buffer pH 7.5, 25 mM $MgCl_2$, 100 μM resorufin and 1 mg cytosolic protein. The reaction was initiated by the addition of 125 μM NADPH and repetitive spectra were recorded every minute. a: A decrease in absorbance at 338 and 572 nm was noticed for NADPH and resorufin, respectively. b: Presence of dicumarol (10 μM) in the reaction mixture showed no change in NADPH and resorufin.

The metabolism of PRF has been shown to be mediated by P-450 IIB1, the inducible form of P-450 [11]. The variety of substrates hydroxylated by P-450 monooxygenase system has contributed to a broad spectrum of catalytic assay techniques. In the recent past most monooxygenase reactions have employed analysis of product through continuous fluorometric assays [19]. The assay described by Lubet et al. [11] for PROD also involves continuous monitoring of fluorescence of the product. However, in the present investigation, kinetics of four compounds in the reaction of microsomal PROD can be measured spectrophotometrically with simultaneous establishment of the stoichiometry of the reaction.

The results in the present investigation clearly indicate a spectrophotometric approach to detect microsomal PROD activity wherein monitoring of NADPH, PRF, resorufin and NADP is successfully attempted along with simultaneous measurement of stoichiometry of enzyme reaction. The method can be easily adopted for other enzyme assays where electron donor, substrate and product have distinct absorption maxima.

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