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Short communication

A Continuous Spectrophotometric Assay for NADPH-cytochrome P450 Reductase Activity Using 1,1-Diphenyl-2-Picrylhydrazyl

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NADPH-cytochrome P450 reductase (CPR) transfers electrons from NADPH to cytochrome P450, and catalyzes the one-electron reduction of many drugs and foreign compounds. Various forms of spectrophotometric titration have been performed to investigate the electron-accepting properties of CPR, particularly, to examine its ability to reduce cytochrome c and ferricyanide. In this study, the reduction of 1,1-diphenyl-2-picrylhydrazyl (DPPH) by CPR was assessed as a means of monitoring CPR activity. The principle advantage of DPPH is that its reduction can be assayed directly in the reaction medium by a continuous spectrophotometry. Thus, electrons released from NADPH by CPR were transferred to DPPH, and DPPH reduction was then followed spectrophotometrically by measuring A_{520} reduction. Optimal assay concentrations of DPPH, CPR, potassium phosphate buffer, and NADPH were first established. DPPH reduction activity was found to depend upon the strength of the buffer used, which was optimal at 100 mM potassium phosphate and pH 7.6. The extinction coefficient of DPPH was 4.09 mM^{-1} cm^{-1}. DPPH reduction followed classical Michaelis-Menten kinetics (K_m = 28 μM, k_{cat} = 1690 min^{-1}). This method uses readily available materials, and has the additional advantages of being rapid and inexpensive.

Keywords: Continuous assay, DPPH, NADPH-cytochrome P450 reductase, Reduction activity

Introduction

Microsomal NADPH-cytochrome P450 reductase (CPR, EC 1.6.2.4) mediates the transfer of electrons from NADPH to cytochrome P450 (P450 or CYP), to other microsomal proteins, to nonphysiologic electron acceptors like cytochrome *c*, potassium ferricyanide, 2,6-dichloroindophenol (DCIP), and to therapeutically important compounds like mitomycin c and benzotriazine SR4233 (Porter, 1991; Sevrioukova and Peterson, 1995). Electron transfer to electron acceptors such as cytochrome *c* or P450 proceeds from NADPH to FAD and FMN, while ferricyanide and 3-acetylpyridine adenine dinucleotide phosphate (AcPyrADP) accept electrons directly from FAD (Iyanagi and Mason, 1973; Vermilion *et al*., 1981; Kurzban and Strobel, 1986). Many important proteins also accept electrons from CPR for their physiological functions, e.g. cytochrome b_5 (Enoch and Strittmatter, 1979), heme oxygenase (Schacter *et al*., 1972), and squalene epoxidase (Ono *et al*., 1977). CPR has also been shown to initiate lipid peroxidation by the one-electron reduction of molecular oxygen (Sevanian *et al*., 1990).

1,1-Diphenyl-2-picrylhydrazyl (DPPH) reacts with thiols and mercaptans, amines, phenols, acids, and hydroaromatic compounds (Goupy *et al*., 2003). This property is widely used in analytical practice for quantitative and semi-quantitative determinations. DPPH has also been used as a free-radical scavenger. Moreover, the stable free radical of DPPH is one of the most widely used substances as a primary standard in quantitative EPR spectrometry.

Relatively few attempts have been made to develop a method for the quantitative determination of DPPH. The total free radical scavenger capacity of edible oils was determined spectrophotometrically by measuring the disappearance of the DPPH radical (Espín *et al*., 2000). The reaction involves a color change from violet to yellow, which can easily be monitored by following absorbance at 520 nm.

We report here upon the development and characterization of a continuous spectrophotometric assay for the reduction of DPPH by CPR. The reaction scheme is shown in Fig. 1. The absorption spectrum of DPPH differs from that of NADPH (a required co-factor), allowing the disappearance of DPPH to be monitored directly in a cuvette.

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Materials and Methods

Materials Cytochrome c, potassium ferricyanide, DPPH, β-NADPH, Tris, and HEPES were obtained from the Sigma Chemical Co (St. Louis, USA). All other chemicals were of analytical grade. Rat recombinant CPR was expressed in *Escherichia coli* and purified as previously described (Hanna *et al*., 1998).

Apparatus Absorption spectra were recorded using a Shimadzu UV-1601 spectrophotometer (Tokyo, Japan). Spectrophotometric measurements between 290-700 nm were performed using standard 1 cm disposable cuvettes. All measurements were performed at 23°C.

Determination of the extinction coefficient (ε) of DPPH All solutions were prepared fresh daily and kept in the dark to avoid DPPH decomposition. DPPH solutions (10 mM) were prepared in absolute ethanol and then rapidly diluted to the indicated concentrations. Spectrophotometric measurements at A_{520} were then performed to determine the extinction coefficient of DPPH.

Enzymatic activities of NADPH-cytochrome P450 reductase (CPR) All continuous spectrophotometric assays for CPR activity were performed using standard 1 cm disposable cuvettes in a total reaction volume of 1 ml. Cytochrome *c* reduction activity was measured as previously described (Shen and Kasper, 1995) using 40 μM of cytochrome *c* and 5 pmol of CPR in 300 mM potassium phosphate (pH 7.6). Increased absorbance at the wavelength of 550 nm was followed after adding 100 μM NADPH. Cytochrome *c* reduction rates were calculated using ε_{550} = 21.0 $mM^{-1}cm^{-1}$ for reduced cytochrome *c* (Vermilion *et al*., 1981).

The CPR-mediated reduction of ferricyanide was carried out using 500 μM ferricyanide and 5 pmol CPR in 100 mM potassium phosphate (pH 7.6). Reduced absorbance at 420 nm was determined after adding 100 μM NADPH. Ferricyanide reduction rates were calculated using ε_{420} = 1.02 $mM^{-1}cm^{-1}$ for the ferricyanide reduction product (Schellenberg and Hellerman, 1958).

DPPH reductase activity was determined using 300 μM DPPH and 5 pmol CPR in 100 mM potassium phosphate (pH 7.6). Absorbance reductions at 520 nm were determined after adding 100 μM NADPH. An extinction coefficient of 4.09 $mM^{-1}cm^{-1}$ was used to calculate the number of moles of DPPH reduced per mol of enzyme.

Determination of kinetic parameters Reaction mixtures included 5 pmol of CPR in 100 mM of potassium phosphate, and varying concentrations of substrate (1-500 μM potassium ferricyanide and 1-100 μM DPPH) in a total volume of 1 ml. Reactions were initiated by adding 100 μM NADPH. Kinetic parameters (K_m and k_{cat}) were determined by nonlinear regression using Graph-Pad Prism software (San Diego, USA).

Optimal reaction conditions The effect of buffer composition on DPPH reduction was examined using: 100 μM DPPH and 5 pmol CPR in 100 mM of potassium phosphate (pH 7.6), Tris-HCl (pH 7.6), or HEPES (pH 7.6), respectively. A_{520} was determined after adding 100 μM NADPH. The concentration of DPPH was determined spectrophotometrically at 520 nm using a molar extinction coefficient of 4.09 $mM^{-1}cm^{-1}$.

The relation between DPPH reduction and potassium phosphate concentration was examined at constant concentrations of CPR (5 pmol) and DPPH (100 μM), and varying concentrations of potassium phosphate buffer, pH 7.6 (50-500 mM). The reaction was initiated by adding 100 μM NADPH and the reduction of DPPH was monitored at 520 nm.

Absorption spectra of DPPH Two cuvettes were filled with 100 mM potassium phosphate (pH 7.6) and baseline buffer light absorptions in the dual-beam spectrophotometer were recorded from 700 nm to 290 nm. After replacing the buffer in the sample cuvette with the same buffer containing 100 μM DPPH, this spectrum was monitored until no further change occurred. 5 pmol of CPR was then added to the sample cuvette and the spectrum recorded. 100 μM NADPH was then added in the sample cuvette and spectra were recorded at 5-min intervals until no further reduction was observed.

Results and Discussion

Extinction coefficient (ε) of DPPH The stock solution of DPPH was prepared in ethanol to a final concentration of 10 mM and rapidly diluted when required to the required concentration. Measurements at A_{520} were performed using a spectrophotometer using standard disposable cuvettes to determine the extinction coefficient of DPPH, which was found to be 4.09 $mM^{-1}cm^{-1}$ (Fig. 2a).

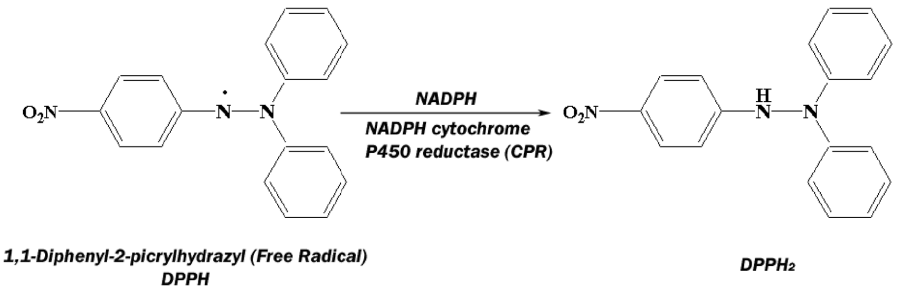

Fig. 1. Reduction of DPPH catalyzed by CPR in the presence of NADPH. Chemical structures of DPPH and $DPPH_2$.

Table 1. NADPH-cytochrome P450 reductase-catalyzed enzyme activities

Substrate	Reduction rate[a] (nmol product/min/nmol CPR)	k_{cat} (min^{-1})	K_m (μM)
Cytochrome *c*	3870 ± 20[b]	ND[c]	ND[c]
Ferricyanide	1890 ± 20	1860 ± 110	9.4 ± 3.4
DPPH	1640 ± 10	1690 ± 90	28 ± 6

[a]Reaction rates were determined in 100 mM potassium phosphate, pH 7.6, in the presence of 100 mM NADPH, at the following electron acceptor concentrations: cytochrome *c*, 40 μM; potassium ferricyanide, 500 μM; and DPPH, 100 μM. The following extinction coefficients, given as $mM^{-1}cm^{-1}$, were used to calculate the rates of reduction of the substrates: cytochrome *c*, 21.0 at 550 nm (Vermilion *et al*., 1981); ferricyanide, 1.02 at 420 nm (Schellenberg and Hellerman, 1958); and DPPH, 4.09 at 520 nm. Kinetic parameters (k_{cat} and K_m) were determined at various ferricyanide and DPPH concentrations in the presence of 100 μM NADPH.
[b] The results shown are Means ± SD of three independent experiments.
[c]ND, not determined.

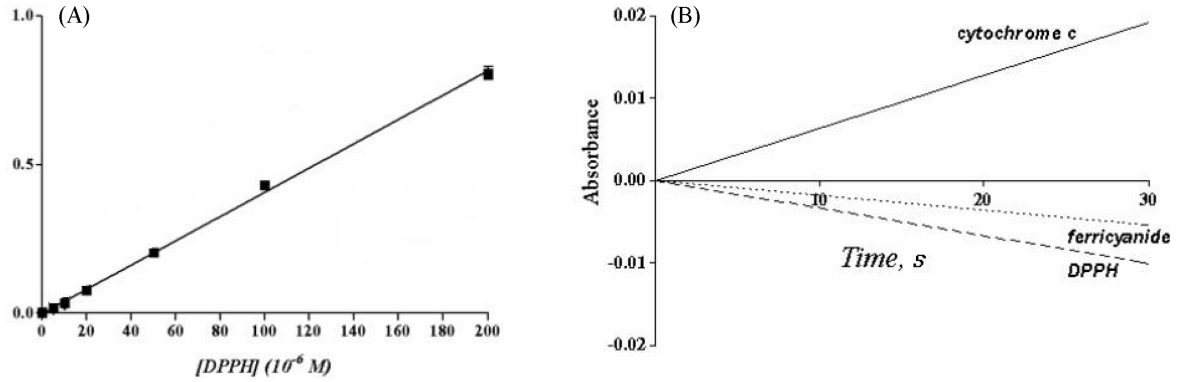

Fig. 2. (A) Standard plots for the determination of the extinction coefficient (ε) of DPPH and (B) the kinetics of CPR catalyzed cytochrome *c* (—), potassium ferricyanide (· · ·), and DPPH (- - -) reduction, by NADPH. The extinction coefficient was found using Beer's Law, A = εcl, where ε is the extinction coefficient, c the concentration, and l the cuvette path length (1.0 cm). ε is represented by the slope of the plot of A *versus* concentration in mol/L. The vertical axis represents absorbance (*A*). The CPR-catalyzed reduction of cytochrome *c*, potassium ferricyanide, and DPPH, monitored at 550 nm, 420 nm, and 520 nm, respectively, in the presence of NADPH. Linear regression slopes were determined using Graph-Pad Prism software (San Diego, USA). Experimental conditions were as described in "Materials and Methods".

Enzyme assays The CPR-catalyzed NADPH-dependent reductions of cytochrome *c*, potassium ferricyanide, and DPPH are shown in Fig. 1b and summarized in Table 1. The turnover numbers of for the reduction of cytochrome *c*, ferricyanide, and DPPH were 3870, 1890, and 1640, respectively (Table 1). These results are consistent with those obtained for CPR purified recombinant rat enzyme expressed in *E. coli* (Shen and Kasper, 1995).

Determination of kinetic parameters Kinetic analyses were carried out with potassium ferricyanide and DPPH as electron acceptors, and results are summarized in Table 1. Although the K_m value of DPPH for CPR was found to be three times higher than that of ferricyanide, the k_{cat} of DPPH was 1690 min^{-1}, which was comparable to that of ferricyanide (1860 min^{-1}). This result suggests that DPPH can be used as a substrate for the CPR assay at 520 nm.

Optimal conditions to assay DPPH reduction by CPR To establish the optimal conditions for the CPR-catalyzed NADPH-dependent DPPH reduction, the effect of buffer composition on DPPH reduction was examined in the presence of; 100 mM Tris-HCl (pH 7.6), potassium phosphate (pH 7.6), or HEPES (pH 7.6) (Fig. 3a). The concentrations of CPR, DPPH, and NADPH were fixed. The effect of the potassium phosphate (pH 7.6) concentration was also examined (Fig. 3b). DPPH reduction activity decreased as the concentration of potassium phosphate (pH 7.6) increased. The activity of DPPH reduction in Tris-HCl (pH 7.6) was lower than in potassium phosphate (pH 7.6) or HEPES (pH 7.6). In accord with these results, 100 mM of potassium phosphate buffer (pH 7.6) was chosen and used routinely to assay DPPH reduction activity.

Spectral analyses The visible absorption spectra of DPPH were measured to examine the reduction of DPPH by CPR in the presence of NADPH (Fig. 4), whereas two peaks of DPPH at 335 and 535 nm reduced, a new band was observed due to the formation of reaction products (in the range of 385-480 nm).

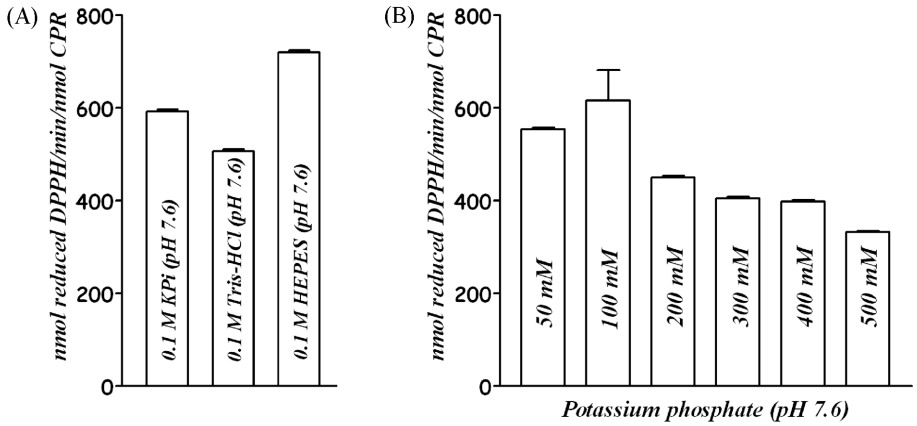

Fig. 3. (A) The buffer-dependent reduction of DPPH by CPR. The reduction of DPPH was measured using 5 pmol of CPR, 100 μM DPPH, 100 μM NADPH in 100 mM potassium phosphate buffer (pH 7.6), Tris-HCl (pH 7.6), or HEPES (pH 7.6). (B) The relation between DPPH reduction and potassium phosphate concentration. The reduction of DPPH was measured using 5 pmol CPR and 100 μM of DPPH in the presence of NADPH (100 μM), potassium phosphate (pH 7.6) concentrations varied from 50 mM to 500 mM.

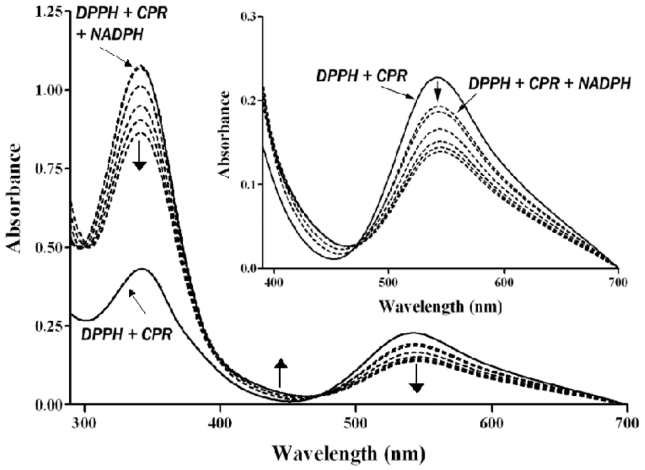

Fig. 4. Consecutive spectra, obtained for DPPH reduction by NADPH-cytochrome P450 reductase (CPR). The assay medium (1.0 mL) contained 100 μM DPPH, 5 pmol NADPH CPR, and 100 μM NADPH in 100 mM potassium phosphate (pH 7.6). As the two absorption peaks of DPPH at 335 and 535 nm decreased, a new band appeared due to the formation of reaction products (in the range of 385-480 nm). Isosbestic points were observed at 385 and 480 nm.

In summary, we describe a continuous spectrophotometric method for determining the activity of NADPH-cytochrome P450 reductase (CPR). The extinction coefficient of DPPH at 520 nm (ε_{520} = 4.09 $mM^{-1}cm^{-1}$) is 4-fold higher than that of ferricyanide (ε_{420} = 1.02 $mM^{-1}cm^{-1}$). Because the turnover number for DPPH reduction was comparable to that of ferricyanide reduction, DPPH may be used for the continuous spectrophotometric assay of CPR. This assay has the advantage of using commercially available inexpensive substrates. In addition, the described procedure offers straightforward and rapid means of determining CPR activity.

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