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Short communication

A Continuous Spectrophotometric Assay for NADPH-cytochrome P450 Reductase Activity Using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide

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NADPH-cytochrome P450 reductase (CPR) transfers electrons from NADPH to cytochrome P450 and also catalyzes the one-electron reduction of many drugs and foreign compounds. Various spectrophotometric assays have been performed to examine electron-accepting properties of CPR and its ability to reduce cytochrome b_5, cytochrome *c*, and ferricyanide. In this report, reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by CPR has been assessed as a method for monitoring CPR activity. The principle advantage of this substance is that the reduction of MTT can be assayed directly in the reaction medium by a continuous spectrophotometric method. The electrons released from NADPH by CPR were transferred to MTT. MTT reduction activity was then assessed spectrophotometrically by measuring the increase of A_{610}. MTT reduction followed classical Michaelis-Menten kinetics (K_m = 20 μM, k_{cat} = 1,910 min^{-1}). This method offers the advantages of a commercially available substrate and short analysis time by a simple measurement of enzymatic activity of CPR.

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

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Introduction

The microsomal NADPH-cytochrome P450 reductase (CPR, EC 1.6.2.4) mediates the transfer of electrons from NADPH to cytochrome P450 (P450 or CYP), other microsomal proteins, and cytochrome *c*. It also catalyzes the reduction of many drugs and foreign compounds such as potassium ferricyanide, 2,6-dichloroindophenol, 1,1-diphenyl-2-picrylhydrazyl (DPPH), and mitomycin *c* (Sevrioukova and Peterson, 1995; Yim *et al.*, 2004). There are many important proteins that accept electrons from CPR for their physiological functions, e.g. cytochrome b_5, heme oxygenase, and squalene epoxidase. CPR also has been shown to initiate lipid peroxidation by one-electron reduction of molecular oxygen.

Tetrazolium salts are used extensively in cell proliferation and cytotoxicity assays, enzyme assays, histochemical procedures, and bacteriological screening. In each process, tetrazolium salts are metabolically reduced to highly colored end products called formazans (Altman, 1976). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is a monotetrazolium salt. The reduction of MTT is one of the most frequently used methods for measuring cell proliferation and cytotoxicity (Mosmann, 1983).

In this report, reduction of MTT by CPR has been assessed as a method for monitoring CPR activity (Fig. 1). The principle advantage of this substance is that the reduction of MTT can be assayed directly in the reaction medium by a continuous spectrophotometric method. The electrons released from NADPH by CPR were transferred to MTT, and then MTT reduction activity was assessed spectrophotometrically by measuring the increase of A_{610} due to the formation of blue formazan. The extinction coefficient of MTT is 11.3 mM^{-1}cm^{-1} (Prochaska, 1988). MTT reduction followed classical Michaelis-Menten kinetics (K_m = 20 μM, k_{cat} = 1910 min^{-1}) (Table 1). This method offers the advantages of short analysis time and the use of a commercially available substrate.

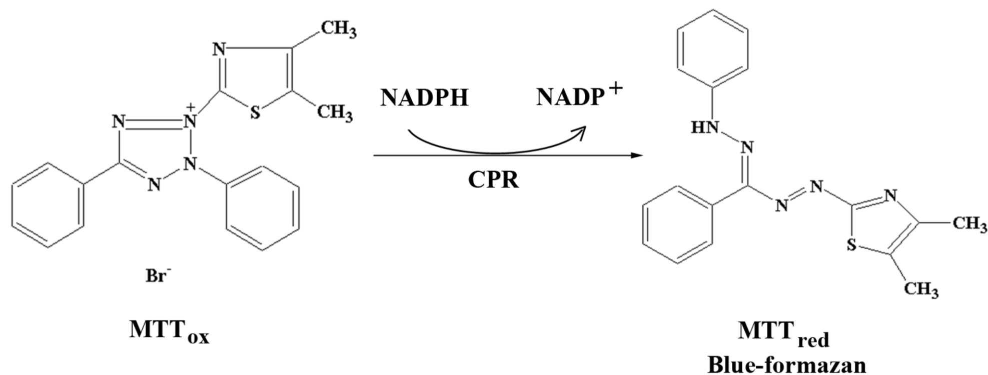

Fig. 1. Reduction of MTT catalyzed by CPR in the presence of NADPH. Chemical structures of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and its reduced product, blue formazan.

Table 1. Reduction of chemical substrates catalyzed by NADPH-cytochrome P450 reductase[a]

Substrate	Typical wavelength (nm) to detect product	k_{cat} (min^{-1})	K_m (μM)	References
MTT	610	1910 ± 40[b]	20 ± 2	This work
Ferricyanide	420	1860 ± 110	9.4 ± 3.4	(Yim *et al.*, 2004)
DPPH	520	1690 ± 10	28 ± 6	(Yim *et al.*, 2004)

[a]Kinetic parameters were determined as described under Materials and methods.
[b]Means ± SD of at least two independent experiments are given.

Materials and Methods

Materials MTT, β-NADPH, β-$NADP^+$, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase 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 described (Hanna, 1998).

Apparatus Absorption spectra were recorded with a Shimadzu UV-1601 spectrophotometer (Tokyo, Japan). The spectrophotometric measurements were performed using standard 1 cm disposable cuvettes. All measurements were performed at 23°C.

Enzymatic activity of NADPH-cytochrome P450 reductase Reactions for measurement of MTT reductase activity were carried out with 1 ml of mixture as follows: 100 μM MTT and 20 pmol CPR in 100 mM potassium phosphate (pH 7.6). The increase of absorbance at 610 nm was determined after the reaction was started by adding an NADPH generating system (final concentrations of 0.5 mM $NADP^+$, 5 mM glucose 6-phosphate, and 0.5 unit of glucose 6-phosphate dehydrogenase/ml). An extinction coefficient of 11.3 $mM^{-1}cm^{-1}$ was used to calculate the number of moles of MTT reduced per mol of enzyme (Prochaska, 1988). All continuous spectrophotometric assays for CPR activity were performed using standard 1 cm disposable cuvettes in a total reaction volume of 1 ml.

Determination of kinetic parameters Reactions for the measurement of kinetic parameters of MTT reduction by CPR were carried out as follows: reaction mixtures included 20 pmol CPR in 100 mM potassium phosphate, and varying concentrations of substrate (0.1-500 μM MTT) in a total volume of 1 ml. Reactions were initiated by the addition of an NADPH generating system, and the increase in A_{610} was monitored for 30 s to calculate the rates. Kinetic parameters (K_m and k_{cat}) were determined using nonlinear regression with Graph-Pad Prism software (San Diego, USA).

Absorption spectra of MTT Two cuvettes were filled with the 100 mM potassium phosphate (pH 7.6) and a baseline of equal light absorption of the buffer in a dual-beam spectrophotometer from 700 nm to 350 nm was recorded. After the buffer in the sample cuvette was replaced with the same buffer containing 100 μM MTT, spectra were monitored until no further change. 20 pmol of CPR was then added to the sample cuvette and the spectra were recorded. An NADPH generating system was added to the sample cuvette to initiate the reduction of MTT and to record spectra at 1-min intervals.

Results and Discussion

The stock solution of MTT was prepared in 10 mM potassium phosphate (pH 7.4) to make a final concentration of 10 mM. It was made fresh daily, kept in an amber vial, and diluted to the indicated concentrations. The CPR-catalyzed reduction of MTT was measured at 610 nm with increasing concentrations of MTT (Fig. 2A). Increase of A_{610} during the first 30 s was used to determine the reduction rate at the indicated concentration of MTT.

Kinetic analysis was carried out with MTT as an electron acceptor and is summarized in Table 1. Kinetic parameters of known chemical substrates for CPR are also shown for

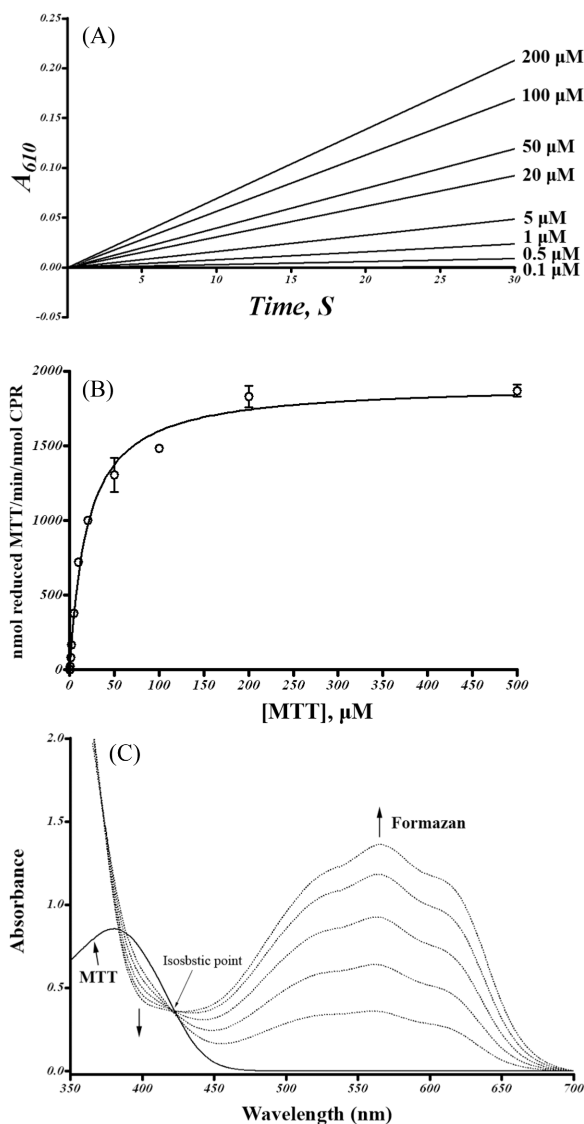

Fig. 2. (A) Concentration-dependent reduction of MTT by CPR. The reduction of MTT was measured with varying concentrations of MTT (0.1-500 μM) in the presence of an NADPH generating system for 30 s. (B) The kinetic parameter of MTT reduction was determined as described in Materials and Methods. Linear regression slopes were determined using linear regression with Graph-Pad Prism software (San Diego, USA). (C) Consecutive spectra, obtained for the MTT reduction by NADPH cytochrome P450 reductase (CPR). After an NADPH generating system was added to the sample cuvette, spectra were recorded at 1-min intervals. Experimental conditions were carried out as described in Materials and Methods.

comparison. The k_{cat} for CPR was measured with MTT (1,910 min^{-1}), and was comparable to that for CPR measured with potassium ferricyanide (1,860 min^{-1}) and DPPH (1,690 min^{-1}). Although the K_m value of MTT (20 μM) for the CPR is ~2-fold higher than that of ferricyanide (9.4 μM), it was comparable to that of DPPH (28 μM). This suggests that MTT can be used as a substrate for the CPR assay at 610 nm. Interestingly, all chemical substrates shown in Table 1 have similar kinetic parameters. Each chemical can be used at different wavelengths depending upon the assay conditions.

The visible absorption spectra were measured to examine the reduction process of MTT by CPR in the presence of NADPH (Fig. 1C). Typical spectra of blue formazan, the reduction product of MTT, appeared due to the formation of reaction products (in the range of 420-700 nm, with three λ_{max} (nm) of the product) depending upon the reaction time. An isosbestic point at 420 nm was observed.

Although the range of wavelength for the MTT assay is similar to that of DPPH assay (Fig. 2C) (Yim *et al*, 2004), MTT has several distinct advantages over DPPH as a substrate for CPR. First, MTT is more stable than DPPH. As DPPH is a radical, DPPH stock solution should be prepared freshly for each activity assay. MTT stock solution is stable at room temperature for several days. Second, MTT is freely soluble up to 5 mM in water whereas DPPH is not soluble in water and the stock solution should be made with an organic solvent such as ethanol. Finally, MTT has an advantage over DPPH for a colorimetric high throughput assay, as the reduction of MTT by CPR shows an apparent color change from yellow to blue, which can be easily observed with the naked eye. On the other hand, when DPPH is used as a substrate for CPR, a decrease of absorbance at 520 nm is typically measured. There are no color changes observable with the naked eye during the DPPH assay.

In summary, we describe a continuous spectrophotometric method to measure the activity of NADPH-cytochrome P450 reductase (CPR) using MTT as a substrate. As the extinction coefficient of the MTT at 610 nm ($\varepsilon_{610} = 11.3\ mM^{-1}cm^{-1}$) is 3-10 fold higher than that of DPPH ($\varepsilon_{520} = 4.09\ mM^{-1}cm^{-1}$) and ferricyanide ($\varepsilon_{420} = 1.02\ mM^{-1}cm^{-1}$), MTT shows adequate sensitivity as a substrate for a CPR activity assay. As the turnover number of MTT was comparable to that of ferricyanide and DPPH, MTT can be used for a continuous spectrophotometric assay of CPR. The assay offers the advantages of use of a commercially available substrate and simple measurement of enzymatic activity of CPR that involves short analysis time.

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