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A continuous fluorimetric assay for protoporphyrinogen oxidase by monitoring porphyrin accumulation

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Abstract

A continuous spectro fluorimetric assay for protoporphyrinogen oxidase (PPO, EC 1.3.3.4) activity has been developed using a 96-well plate reader. Protoporphyrinogen IX, the tetrapyrrole substrate, is a colorless non fluorescent compound. The evolution of the fluorescent tetrapyrrole product, protoporphyrin IX, was detected using a fluorescence plate reader. The apparent K_m (K_{app}) values for protoporphyrinogen IX were measured as 3.8 ± 0.3 , 3.6 ± 0.5 , and 1.0 ± 0.1 μM for the enzymes from human, *Myxococcus xanthus*, and *Aquifex aeolicus*, respectively. The K_i for acifluorfen diphenylether herbicide, was measured as $0.53 \mu\text{M}$ enzyme. Also, the specific activity of mouse liver mitochondrial PPO was measured as $0.043 \text{ nmol h}^{-1}/\text{mg mitochondria}$, demonstrating that this technique is useful for monitoring low-enzyme activities. This method can be used to accurately measure activities as low as 0.5 nM min^{-1} , representing a 50-fold increase in sensitivity over the currently used discontinuous assay. Furthermore, this continuous assay may be used to monitor up to 96 samples simultaneously. These obvious advantages over the discontinuous assay will be of importance for both the kinetic characterization of recombinant PPOs and the detection of low concentrations of this enzyme in biological samples.

Keywords

Heme biosynthesis; Protoporphyrin; Enzyme kinetics; Fluorescence; Herbicide

Protoporphyrinogen oxidase (PPO,¹ EC 1.3.3.4) catalyzes the oxygen-dependent six-electron oxidation of protoporphyrinogen IX to the fully conjugated macro-cycle of protoporphyrin IX [1]. Mutations in the PPO gene may give rise to a condition known as variegate porphyria [2]. In eukaryotic organisms, PPO has been localized to the cytosolic side of the inner

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¹Abbreviations used:

PPO	protoporphyrinogen oxidase
FAD	flavin adenine dinucleotide
MOPS	4-morpholinepropanesulfonic acid
Tris	Tris(hydroxymethyl)aminomethane carbonate
PMSF	phenylmethylsulfonyl fluoride
EDTA	ethylenediamine tetraacetic acid.

mitochondrial membrane and requires molecular oxygen for catalysis to proceed [3-5]. The human, *Bacillus subtilis*, *Myxococcus xanthus*, *Aquifex aeolicus*, and mouse enzymes have previously been cloned, overexpressed in *Escherichia coli*, and purified to homogeneity [6-10]. A noncovalently bound flavin adenine dinucleotide (FAD) cofactor was detected in each of these enzymes, and several were inhibited by acifluorfen, a diphenylether herbicide. Also, a recent study reported that acifluorfen is a competitive inhibitor of human PPO [11].

The first assays of PPO exploited the change in absorbance elicited by the accumulation of protoporphyrin IX [12]. The enzymatic rate was calculated by measuring the difference between the Q-band absorption peaks of protoporphyrin IX (450-700 nm) before and after catalysis. This was found to be preferable to an alternate procedure involving the spectrophotometric detection of protoporphyrin IX in acid solution under anaerobic conditions that caused rapid autooxidation of the protoporphyrinogen substrate [13].

The discontinuous spectrophotometric technique [12] was later modified to monitor the evolution of protoporphyrin IX fluorescence [14]. Although this improved sensitivity, the assay remained discontinuous. The reaction conditions of this discontinuous fluorescence assay were identical to those used in the discontinuous spectrophotometric assay except that Tween was included to enhance protoporphyrin IX fluorescence. The inclusion of glutathione was intended to help reduce the autooxidation of the protoporphyrinogen IX substrate, although this had been shown to inhibit PPO activity from certain microbial sources [15]. Also, chelating agents were used to prevent the insertion of divalent cations into the tetrapyrrole ring because ferrochelatases in the cell extracts may incorporate zinc or ferrous iron. This is no longer necessary when purified recombinant PPOs are assayed.

Both of these assay procedures were subsequently assessed [16]. Poulson's discontinuous spectrophotometric assay [12] was found to have a limit of sensitivity of 56 nM min^{-1} , whereas Brenner's discontinuous fluorescence assay [14] was slightly more sensitive, exhibiting a lower limit of 28 nM min^{-1} . The major shortcomings of these assays are a lack of sensitivity for the detection of low-enzyme activities in biological samples and the autooxidation of substrate. In this article, we describe a continuous fluorescence assay that detects PPO activities as low as 0.5 nM min^{-1} . Multiple samples and controls may be monitored simultaneously, facilitating the subtraction of spontaneous protoporphyrinogen IX oxidation. Also, by increasing the wavelength and reducing the intensity of excitation light, we have managed to greatly reduce this autooxidation. We compare data obtained using the continuous and discontinuous methods and highlight the benefits of the continuous assay for use in clinical diagnosis and enzyme kinetics alike.

Materials and methods

Materials

Protoporphyrin IX was purchased from Porphyrin Products (Logan, UT, USA). Tween 20 was obtained from EM Science (Gibbstown, NJ, USA). 4-Morpholinepropanesulfonic acid (MOPS) was purchased from Fisher Scientific (Pittsburgh, PA, USA). Tris(hydroxymethyl) aminomethane carbonate (Tris), potassium chloride, sodium dihydrophosphate, and sodium metal were purchased from J.T. Baker. The remaining chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

Protein expression and purification

Recombinant PPOs were overexpressed and purified as reported previously [7,9,10]. *E. coli* JM109 cells were transformed, and the cells were grown overnight at 30°C . The growth media were supplemented with ribo-flavin at a final concentration of $0.75 \text{ }\mu\text{gml}^{-1}$ 2h prior to

harvesting. The cells from 1 L of cell culture were collected by centrifugation and resuspended in buffer (50 mM Tris/MOPS (pH 8.0), 100 mM KCl, 0.5% Tween 20 (v/v)) containing 1 mg ml⁻¹ phenylmethylsulfonyl fluoride (PMSF), sonicated on ice for 60 s, and centrifuged at 100,000g for 30 min to remove membranes and insoluble proteins. The supernatant was applied to Talon (Clontech, Palo Alto, CA, USA) chelate columns (bed volume 3 ml), which were subsequently washed with 20 column volumes of buffer and a further 20 volumes of buffer containing 15 mM imidazole. The purified proteins were eluted with buffer containing 250 mM imidazole. The imidazole was subsequently removed using a Biogel (Bio-Rad, Hercules, CA, USA) desalting column with a bed volume of 30 ml. Protein preparations were analyzed using UV-visible spectroscopy to confirm the presence of the noncovalently bound FAD cofactor. Protein concentrations were determined spectrophotometrically using calculated extinction coefficients based on the amino acid composition. These ϵ_{270} values were 48.0, 21.9, and 34.6 mM⁻¹ cm⁻¹ for enzymes from human, *M. xanthus*, and *A. aeolicus*, respectively.

Preparation of mouse liver mitochondria

A 1.6-g liver from a C57BL/6x inbred mouse was homogenized in 8 ml of ice-cold homogenization buffer (10 mM Tris-HCl (pH 8.0), 0.25 M sucrose, 1 mM ethylenediamine tetraacetic acid (EDTA), 0.2 mg ml⁻¹ PMSF). The homogenized sample was aliquotted into 1.5-ml Eppendorf tubes on ice. The samples were centrifuged at 10,000g for 10 min at 4°C. The supernatants were decanted, and the pellets were weighed. The pellets were resuspended in ice-cold PPO assay buffer (50 mM NaH₂PO₄ (pH 8.0), 0.2% Tween 20 (v/v)) to give a concentration of 273 mg mitochondria/ml, of which 8 to 25 μ l was used in the kinetic assays.

Porphyrin and inhibitor preparation

Protoporphyrin IX (3 mg) was solubilized with four drops of 30% ammonium hydroxide and dissolved in 2.5 ml of freshly prepared 10 mM KOH containing 20% ethanol. This was then diluted with 2.5 ml of 10 mM KOH solution. Sodium amalgam was prepared, as described previously [14,17], by mixing 3.5 g of sodium metal with 75 g of mercury under a stream of nitrogen in a fume hood. It was important to ensure that all of the oil was removed from the sodium metal before mixing. The hot amalgam was cooled to room temperature, broken into 1-cm³ pieces, and stored under nitrogen for up to 1 week. Immediately prior to use, 20 g of amalgam was pulverized with a mortar and pestle and was transferred to a flask under nitrogen in the dark. The 5-ml solution of protoporphyrin IX was transferred to the flask. The flask was sealed and shaken until the solution became colorless, making sure to vent any evolved gas. The pH was adjusted to 8.0 using a solution of 2 M MOPS. The resultant protoporphyrinogen solution was filtered (using a 0.2- μ m syringe filter), placed in the dark under nitrogen, and used immediately.

In assays where acifluorfen was present (Chem Services), the acifluorfen was added from a stock solution in dimethyl sulfoxide. The addition of an equivalent volume of dimethyl sulfoxide alone had a negligible effect on PPO catalysis. All assay procedures were carried out in a darkened room. Concentrations of protoporphyrin IX were determined in 2.7 N HCl using ϵ_{557} 20.7 mM⁻¹ cm⁻¹ [13].

PPO assays

PPO activity was monitored using a continuous assay via the detection of porphyrin fluorescence. This was performed on a Synergy HTI plate reader using 528 $\pm$ 20-nm and 545 $\pm$ 40-nm bandpass filters and a 550-nm highpass filter for the excitation light. A 635 $\pm$ 32-nm bandpass filter was used on the emission side. The excitation Soret band for protoporphyrin IX at 406 nm was not used because this caused saturation of the photomultiplier. Three Wlters were used for the excitation light because a reduction in light intensity greatly reduced the autooxidation of protoporphyrinogen IX.

The instrument was calibrated with known concentrations of protoporphyrin IX. A linear relationship between fluorescence and [protoporphyrin IX] was achieved up to 2.5 μM porphyrin. An opaque, clear-bottom, 96-well, full-diameter plate (Corning, Corning, NY) was used for the assays. The reaction mixtures were preincubated at 37 °C in the dark for 3 min. The reactions were started by the addition of PPO, which was also preincubated at 37 °C, and the evolution of protoporphyrin IX was monitored for 30 min. The reaction mixtures for the *A. aeolicus* enzyme were preincubated and monitored at 50 °C, the maximum temperature for the plate reader. The nonenzymatic rates were determined concomitantly and were subsequently subtracted. These background rates were typically less than 5% of the enzyme-catalyzed rates. Wells containing heat-inactivated samples were used as controls for the mouse liver mitochondria assays. The mitochondrial extract was heated at 85 °C for 20 min and was assayed for PPO activity in parallel with the other samples. On the addition of the mitochondrial extract, the reaction mixture became slightly turbid. However, this did not seem to affect the detection of protoporphyrin IX fluorescence. Reaction conditions and concentrations of reactants/ inhibitors are quoted in the figure legends. The total volume of the reaction mixture was 100 μl and contained 50 mM NaH_2PO_4 (pH 8.0), 0.2% Tween 20 (v/v), and 2.5 glutathione. The mouse liver mitochondria were assayed in the presence of 2.5 mM EDTA. The concentration of protoporphyrinogen IX was determined retrospectively after the complete autooxidation of the stock solution to protoporphyrin IX, which was quantified spectrophotometrically in 2.7 N HCl [13]

Analysis of kinetic data

Steady-state rates were estimated by fitting the time courses to straight lines using linear regression. Some traces exhibited small burst phases (data not shown) or lag phases. The steady-state rate was taken as the maximum rate that followed these burst/lag phases. Steady-state rate data (v vs. $[\text{S}]$) were fitted to Eq. (1) (using unweighted nonlinear regression in Sigma Plot 8 of SPSS)

$$v = \frac{V_{\text{app}}[\text{S}]}{K_{\text{app}} + [\text{S}]}, \quad (1)$$

where v is the rate, V_{app} is the apparent maximum rate, K_{app} is the apparent Michaelis constant, and $[\text{S}]$ is the substrate concentration. Error bars were not included because the estimates of the steady-state rates were obtained from linear regression analysis, where r^2 values were greater than 0.95 (data not shown).

IC_{50} values were calculated by fitting v versus [inhibitor] data to a single binding site model described by Eq.(2)

$$y = \min + \frac{\max - \min}{1 + 10^{x - \log \text{IC}_{50}}}, \quad (2)$$

where y is the percentage of maximal rate, max and min are the y values at which the curve levels off, x is the logarithm of inhibitor concentration, and IC_{50} is the inhibitor concentration that elicits 50% of the total inhibition.

Calculated K_i values were obtained by applying the following relationship, which exists for competitive inhibition among K_i , K_m , and IC_{50} at any saturating substrate concentration (S)

$$K_i = \frac{\text{IC}_{50}}{S / K_m + 1}. \quad (3)$$

Absorbance and fluorescence spectroscopy

Absorbance spectra were recorded on a Cary 50 (Varian) spectrophotometer in 50 mM NaH_2PO_4 (pH 8.0), 0.2% Tween 20 (v/v), and 2.5 mM glutathione. Fluorescence excitation and emission spectra were recorded at 25 °C with a Shimadzu RF-5301PC spectrofluorimeter. All PPO samples were in 50 mM NaH_2PO_4 (pH 8.0), 0.2% Tween 20 (v/v), and 2.5 mM glutathione. Samples (3 ml) were incubated for at least 3 min at 25 °C before spectra were recorded. Buffer-only spectra were recorded and subsequently subtracted using Datamax. For emission spectra, an excitation wavelength of 406 nm was used, with excitation and emission slit widths corresponding to 1.5 and 10 nm, respectively. For excitation spectra, an emission wavelength of 632 nm was used, with excitation and emission slit widths corresponding to 10 and 1.5 nm, respectively. In addition, a 345-nm highpass filter was placed at the emission side of the cuvette holder to reduce the detection of scattered excitation light and to eliminate harmonic effects.

Results and discussion

The continuous assay in this study exploits the fluorescent properties of protoporphyrin IX and the lack of absorption and fluorescence of the tetrapyrrole substrate, protoporphyrinogen IX. Under the assay conditions described, and using an excitation wavelength of 406 nm, the emission maximum for protoporphyrin IX was 632 nm (Fig. 1B). The fluorescence excitation spectrum for protoporphyrin IX was obtained using an emission wavelength of 632 nm (Fig. 1A). The Soret region around 400 nm was not used for the kinetic assay because the resultant high fluorescence intensity caused saturation of the photomultiplier tube. Also, this lower wavelength, higher energy radiation caused rapid autooxidation of the protoporphyrinogen IX substrate. To combat these problems, three filters were used to generate a low-intensity excitation beam at approximately 550 nm. Fluorescence intensity exhibited a linear relationship between 0 and 2.5 μM protoporphyrin IX (data not shown). Furthermore, when 50 nM human PPO was assayed with 3 μM protoporphyrinogen IX, the nonenzymatic rate was found to be negligible in comparison (Fig. 1C). However, higher concentrations of protoporphyrinogen IX exhibit higher autooxidation rates, so nonenzymatic rates must be monitored concomitantly. This is easily achieved using the plate reader because one can monitor 32 assays simultaneously, sampling the fluorescence intensity for each well every 16 s. The minimum interval between time points increases if more wells are used, so usually only one-third of the plate is used. The data points in Fig. 1C were taken at 16-s intervals. The oxidation rate may be estimated far more accurately by using this technique than by estimating a rate from four time points as was necessary previously with the discontinuous assays. The autooxidation rate in Fig. 1C (0.5 nM min^{-1}) was fitted to a straight line using linear regression (Sigma Plot), and the resultant line of best fit had an r^2 value of 0.91. This oxidation rate represents the limit of sensitivity for the continuous fluorescence assay. This technique is far more sensitive than the previous discontinuous fluorescence method, which could be used to measure rates only as low as 28 nM min^{-1} (5 nmol h^{-1} in a 3-ml cuvette) [16].

At low concentrations of protoporphyrinogen IX, the reaction rate slows down as the substrate is exhausted. This is easily observed using the continuous fluorescence method, although such observations might not be immediately apparent using the discontinuous assays, resulting in an underestimation of catalytic rate.

Having established the reaction conditions, we tested the range of enzyme concentrations that could be used with this assay. The evolution of protoporphyrin IX fluorescence was measured for several concentrations of human PPO, and the enzymatic rate was linearly dependent on the enzyme concentration over the range of 25–250 nM (Fig. 2). This demonstrates that the concentration of enzyme typically used (50 nM) is within the linear range and that one can use protein concentrations more than two orders of magnitude below the K_{app} for substrate.

The current assay was assessed by determining the steady-state parameters of purified recombinant PPOs from human, *M. xanthus*, and *A. aeolicus* sources. These data are shown in Fig. 3, and the curves were fitted to the Michaelis equation (Eq. 1)) using nonlinear regression. The kinetic parameters, along with those obtained previously for these three enzymes [7,9, 10], are summarized in Table 1. The V_{app} and K_{app} values are similar to those obtained previously, and the average standard errors for the three plots were 4.1 and 10.6% of the V_{app} and K_{app} values, respectively. This demonstrates that one can quickly and accurately estimate the kinetic parameters for this enzyme without the need for excessive repetition and averaging. However, if averaging is required for enzymes with lower activities, v versus $[S]$ curves may be obtained in triplicate in a single 30-min assay.

The applicability for this continuous fluorescence assay for inhibition studies with human PPO was tested with acifluorfen, a competitive inhibitor with respect to the tetrapyrrole substrate [11]. Human PPO was assayed with varying concentrations of acifluorfen, and the data were fitted to Eq. (2) using nonlinear regression (Fig. 4). The concentration of acifluorfen that elicited a 50% reduction in catalytic rate was $1.48 \pm 0.20 \mu\text{M}$. This compares with $4.2 \pm 0.39 \mu\text{M}$, as reported in a previous study [11]. Using Eq. (3), the apparent K_i was calculated as $0.53 \mu\text{M}$ acifluorfen, which compares with $0.23 \mu\text{M}$, as determined previously [11].

PPO from mouse liver mitochondria was assayed to demonstrate that the continuous fluorescence assay can be used with biological samples. Fig. 5 depicts several PPO assays using mitochondrial concentrations in the range of $22\text{--}68 \text{ mg ml}^{-1}$. Background oxidation was monitored using heat-inactivated samples, and these data have been subtracted. The curves possess a small lag phase, which may be difficult to detect if only four time points are used, as in the discontinuous assay. The steady-state rate was taken as the maximum rate that follows the lag phase. This highlights how useful these progress curves can be when monitoring a nonlinear reaction time course. The Fig. 5 inset demonstrates that enzyme activity is directly proportional to the amount of mitochondria used. The specific activity ($0.043 \text{ nmol h}^{-1}/\text{mg}$ mitochondria) is very low, probably because the samples were not sonicated. However, this is ideal for demonstrating the sensitivity of the continuous fluorescence method. For example, the enzymatic rate with 2.2 mg of mitochondria was 0.12 nmol h^{-1} (20 nM min^{-1}). This activity is below the limit of sensitivity of the discontinuous fluorescence assay (28 nM min^{-1} [16]), demonstrating that the current method may be used to accurately measure low enzymatic rates from biological samples.

Overall, the continuous fluorescence assay offers several significant advantages over existing procedures. The steady-state rates can be determined from the slopes of progress curves, eliminating the problem of nonlinearity in velocity calculations. The use of a lower energy excitation beam around 550 nm reduces the rate of substrate autooxidation and prevents saturation of the photomultiplier at higher protoporphyrin IX concentrations. Another advantage of this assay is that, unlike the discontinuous assays, it does not require one to work for extended periods in the dark. The samples may be loaded onto the micro-plate using a multipipetter under reduced light conditions. In addition, all of the reactions are started at the same time, reducing the risk of autooxidative depletion of the tetrapyrrole substrate before the addition of enzyme.

In summary, the continuous fluorescence assay provides a sensitive method to measure PPO activity and can be used for inhibition studies. Because the procedure may be used to detect small changes in protoporphyrin IX levels in biological samples, it will be useful for assessing diminished PPO activities in variegate porphyria patient samples.

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Fig. 1.

Fluorescence spectra of protoporphyrin IX and a typical kinetic assay: Excitation (A) and emission (B) spectra of protoporphyrin IX under assay conditions. Excitation and emission wavelengths were set to 406 and 632 nm, respectively. Protoporphyrin IX (1 μM) was prepared in assay buffer as described in Materials and methods. (C) Typical assays in the presence (●) and absence (○) of 50 nM human PPO. Enzymatic rate 5880 FU min⁻¹ (which translates to 155 nM min⁻¹). Nonenzymatic rate = 0.54 nM min⁻¹. These rates were estimated using linear regression, with r^2 values for the enzymatic and nonenzymatic fits being 0.999 and 0.911, respectively.

Fig. 2. Dependence of the initial rate of protoporphyrin IX evolution on PPO concentration. Initial rates were determined using 5 μ M protoporphyrinogen IX, and PPO concentrations were 25 nM ($\bullet$), 50 nM ($\circ$), 100 nM ($\blacktriangledown$), 150 nM (∇), and 250 nM ($\blacksquare$). The inset depicts the enzymatic rate versus the protein concentration ($\square$).

Fig. 3.

Determination of apparent Michaelis constants for various protoporphyrinogen oxidases. PPO (50 nm) from human (●), *M. xanthus* (○), and *A. aeolicus* (▼) was assayed using varying concentrations of protoporphyrinogen IX. The human and *M. xanthus* enzymes were assayed at 37 °C, and the hyperthermophilic *A. aeolicus* enzyme was assayed at 50° C. The data were fitted to single rectangular hyperbolae using nonlinear regression, and the kinetic parameters are shown in Table 1.

Fig. 4.

Acifluorfen inhibition of PPO. Human PPO (50nm) was preincubated for 5min at 37°C in the presence of varying concentrations of acifluorfen, a diphenylether inhibitor. Reactions were started by the addition of 6.9μM protoporphyrinogen IX. Reaction rates are shown as the percentages of the catalytic rate in the absence of inhibitor. The data were fitted to Eq. (2) using nonlinear regression. The inhibitor concentration that elicits 50% of the total change in activity is $1.48 \pm 0.20 \mu\text{M}$. The calculated apparent K_i , using Eq. (3), was $0.53 \mu\text{M}$.

Fig. 5. PPO activity of mouse liver mitochondria. Steady-state rates were taken as the maximum rates. Protoporphyrinogen IX ($5.6 \mu\text{M}$) was used in each assay. Background rates (of assays incorporating thermally inactivated samples) have been subtracted. Assays were performed with 2.2 mg (∇), 2.7 mg ($\blacksquare$), 4.1 mg ($\square$), 5.5 mg ($\blacklozenge$), and 6.8 mg ($\blacklozenge$) of mitochondria. The inset depicts the enzymatic rate versus the mass of mitochondria ($\bullet$).

Table 1

Comparison of continuous and discontinuous assays in determination of PPO activity

PPO protein	Continuous assay		Discontinuous assay		Reference
	K_{app} (μ M)	V_{app} (μ M min ⁻¹ / μ M protein)	K_{app} (μ M)	V_{app} (μ M min ⁻¹ / μ M protein)	
H. sapiens	3.8 $\pm$ 0.3	5.7 $\pm$ 0.2	1.7	10.5	[10]
M. xanthus	3.6 $\pm$ 0.5	3.1 $\pm$ 0.2	1.6	5.2	[9]
A. aeolicus	1.0 $\pm$ 0.1	0.4 $\pm$ 0.009	2.	-	[7]