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Methoxy-resorufin ether as an electrochemically active biological probe for cytochrome *P*450 *O*-demethylation

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Abstract

This paper describes the utilisation of methoxy-resorufin ether as an electrochemical probe for studying cytochrome *P*450 CYP6G1. Methoxy-resorufin ether is well established as a versatile substrate for cytochrome *P*450, as its demethylated product, resorufin, is a fluorophore. We show that in addition to these established properties, methoxy-resorufin ether also exhibits reversible two electron transfer on glassy carbon and edge plane graphite electrodes. Cyclic voltammetry measurements and differential pulse voltammetry measurements show that methoxy-resorufin ether can be easily detected at low concentrations (down to 200 nM) in a conventional three electrode electrochemical cell. These properties of methoxy-resorufin ether mean that it could be used as an electrochemical probe, to follow the rate of its demethylation by CYP6G1. We show that electrochemical measurements could discriminate between the enzyme activity of protein microsomes taken from two strains of *Drosophila melanogaster* (fruit fly).

Keywords: Cytochrome *P*450; CYP6G1; Electrochemistry; MROD; Resorufin

1. Introduction

Methoxy-resorufin ether is a substrate for cytochrome *P*450 activity measurements. A current example of the utilisation of methoxy-resorufin ether for such studies is presented by McFadyen et al, who studied the activity of CYP1B1 in renal cell carcinoma, although they used the methoxy-resorufin ether analogue, ethoxy-resorufin ether as the substrate, but still detecting the *O*-dealkylated fluorophore product, resorufin [1]. To date, no reports on the electrochemical properties of methoxy-resorufin ether appear in the literature. Hence we present what we believe is the first systematic study of methoxy-resorufin ether as a biologically active redox probe and illustrate its application in the measurement of cytochrome *P*450 mediated *O*-demethylation.

A single cytochrome *P*450, CYP6G1, is responsible for broad spectrum insecticide resistance in the fruit fly *Drosophila melanogaster* [2]. An electrochemical (Differential Pulse Voltammetry) measurement assay was used to follow the metabolism of the small molecule substrate methoxy-resorufin ether to resorufin (*O*-demethylation) by protein microsomes containing cytochrome *P*450 extracted from both insecticide resistant (Hikone-R) and susceptible (Canton-S) strains of *Drosophila*. The rate of substrate metabolism was higher in resistant flies, as predicted, showing that this electrochemical method is a useful alternative to fluorescence for monitoring substrate metabolism by this important enzyme.

The cytochromes *P*450 are a large family of enzymes that metabolize a broad range of xenobiotics in plants, animals and microbes. They play a crucial role in drug

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activation or detoxification in living creatures [3]. The fruit fly *Drosophila melanogaster* forms an important model for the study of *P*450 mediated drug and pesticide metabolism as all 90 *P*450 genes in the genome have been identified and their roles are now addressable via *Drosophila* genetics [4]. Recent studies have shown that a single mutation within a single *P*450 gene (*Cyp6g1*) is responsible for the over-expression of the corresponding enzyme CYP6G1. This *P*450 has unusually broad substrate specificity, conferring resistance to nicotine, DDT and the neonicotinoids which include the current best selling small molecule insecticide in the world, imidacloprid [2,5]. Direct electrochemical measurements of *P*450s have been performed, for example, by attaching to an electrode in a DDAB surfactant film [6,7]. This allows measurement of the electrochemical reduction of the haem centre within the protein. A number of cytochrome *P*450s have been found to exhibit ether *O*-dealkylase activity, including the CYP6G1 and in the human liver the CYP1A2 and CYP3A6 enzymes all show *O*-dealkylase activity for both methoxy-resorufin ether and ethoxy-resorufin ether [8].

Here we show that it is possible to discriminate between protein microsomes extracted from insecticide resistant and susceptible *Drosophila* by following the rate of *O*-demethylation of methoxy-resorufin ether electrochemically. We also present results from a fluorometric assay which shows the rate of production of resorufin (CYP6G1 metabolism product) has the same dependence on *Drosophila* strain as measured electrochemically. Both analytical methods show that the presence of carbon monoxide, or the absence of NADPH, slow down or terminate substrate metabolism, confirming that substrate metabolism is indeed *P*450 mediated since CO deactivates the haem centre of the *P*450 and NADPH is required for the reaction to proceed [9].

Methoxy-resorufin ether is water soluble, electrochemically active, with a reduction potential of −0.19 V vs. Ag|AgCl, showing fully reversible electron transfer on glassy carbon electrode and edge plane graphite electrodes. The structure is shown in Fig. 1. The CYP6G1 mode of action is to de-methylate methoxy-resorufin ether, forming resorufin (a ketone) [2]. Cytochrome *P*450s are believed to dealkylate alkyl ethers by oxidation of the alkylcarbon adjacent to the bridging oxygen, to form either aldehydes or carboxylic acids. [12] Resorufin has an electrochemical reduction potential at more negative potentials (−0.3 V vs. Ag | AgCl) and importantly is also a fluorophore. This therefore provides two parallel methods of following the activity of CYP6G1 in the protein microsome: via the fluorescence response of the dealkylated product and via the electrochemical response of the substrate.

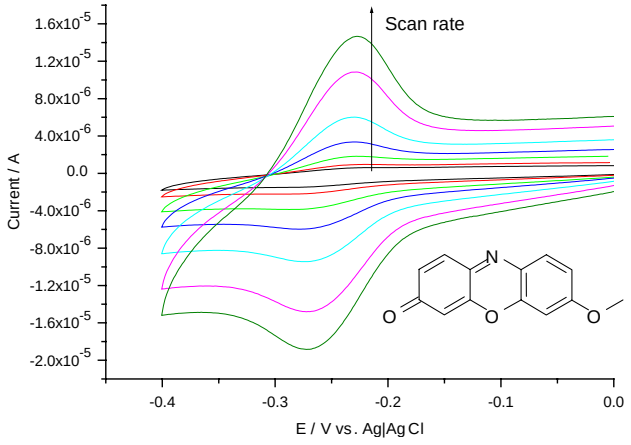

Fig. 1. Cyclic voltammograms of 0.1 mmol dm $^{-3}$ methoxy-resorufin ether, sweep rates at 10, 20, 50, 100, 200, 400 and 600 mV s^{-1}. Note peak separation of ca. 35 mV, and linear relationship between i_p and $\upsilon^{1/2}$. Inset: structure of methoxy-resorufin ether.

2. Materials and methods

2.1. Protein microsome preparation

Drosophila flies were collected at less than 7 days post ecclusion and snap frozen in liquid nitrogen. Approximately 3 ml of *Drosophila* was chilled in liquid nitrogen and the heads were removed using a brass sieve (850 μm aperture) and running fingers over the flies to snap heads off. The abdomens and thoraxes were homogenized in 5 ml ice cold microsome buffer (100 mM potassium phosphate buffer (pH 7.2), 1 mM EDTA, 0.1 mM DTT, 0.4 mM PMSF, all from Sigma) on ice using a hand homogenizer and body parts were removed by filtering through muslin. The body parts were homogenized in a further 5 ml ice cold microsome buffer and the body parts filtered off and discarded. The resulting 10 ml microsome preparation was centrifuged at 10,000 × *g* for 15 min at 4 °C. The supernatant was decanted into an ultracentrifuge tube and centrifuged at 100,000 × *g* for 1 h 15 min at 4 °C. The pellet was re-suspended in 1 ml 100 mM potassium phosphate buffer (pH 7.2), 20% glycerol using a hand homogenizer. Two hundred microliters aliquots were snap frozen in liquid nitrogen and stored at −80 °C.

2.2. Methoxy-resorufin ether electrochemistry

The properties of methoxy-resorufin ether as an electrochemical probe were evaluated using cyclic voltammetry and chronoamperometry. An ethanolic solution of 5 mmol dm^{-3} methoxy-resorufin ether in ethanol was dispersed in phosphate buffered saline pH 7.4 to make a 0.1 mmol dm^{-3} solution in PBS. The solution was degassed under argon. A freshly polished glassy carbon electrode (GCE) of diameter 3 mm, a platinum wire reference electrode and a silver chloride (3 M KCl) reference electrode were employed. Cyclic voltammetry measurements were made by cycling between +0.1 V and −0.4 V vs. Ag|AgCl at scan rates between 10 mV s^{-1} and 600 mV s^{-1}. Finally, a fresh solution of methoxy-resorufin ether was used for a chronoamperometry experiment, with the potential being stepped between −0.1 and −0.35 V vs. Ag|AgCl.

2.3. Real time electrochemistry measurements of methoxy-resorufin ether O-demethylation

The electrochemical measurements of substrate demethylation employed a BAS low volume cell with GCE electrode and an Autolab PGstat 12 potentiostat. The peak reduction current was measured at −0.265 V vs. Ag|AgCl using Differential Pulse Voltammetry (DPV). DPV pulse parameters were: start potential 0.00V, end potential −0.4 V vs. Ag|AgCl. Step potential 1 mV, modulation potential: 50 mV, modulation time: 40 ms, interval time: 100 ms, scan rate 10 mV s^{-1}. The peak current is proportional to the concentration of methoxy-resorufin ether in the solution, since resorufin has a more cathodic reduction potential at −0.32 V vs. Ag|AgCl. Hence, electrochemistry can be used to measure the change in substrate concentration in real-time. [10] A microsome concentration of 2 mg ml^{-1} in pH 7.8 sodium phosphate buffer was used with initial methoxy-resorufin ether concentration at 1 μmol dm^{-3} (in electrochemical cell), with total cell volume of 400 μl. In experiments with the co-factor NADPH, this was added to give a total concentration of NADPH of 2.5 mmol dm^{-3}. In some experiments enzymes were deactivated by bubbling carbon monoxide at ca. 10 ml min^{-1} for 20 min. Total protein concentration was determined using a Bradford assay [11]. Electrochemical measurements were all performed in a minimum of three times and all trends were found to be reproducible. All solutions were degassed in argon for 15 min prior to measurement.

2.4. Fluorescence measurements

A 200 μl reaction was prepared in a black flat bottomed 96-well plate (Sterilin) containing 250 μg microsomes in 100 mmol dm^{-3} pH 7.8 sodium phosphate buffer, 2.5 mmol dm^{-3} NADPH. The reaction is started by the addition of 0.25 mmol dm^{-3} methoxy-resorufin ether dissolved in ethanol. A Labsystems Fluoroskan Ascent plate reader was set to 530 nm excitation and 590 nm emission and the reaction is read for 10 min. The result was compared to a standard curve for resorufin, allowing concentration of the resorufin metabolic product to be determined. Measurements were made on both Canton-S and Hikone-R strains, in the presence and absence of NADPH, and with NADPH but after exposure to carbon monoxide.

3. Results and discussion

3.1. Electrochemistry of methoxy-resorufin ether

Cyclic voltammetry measurements of methoxy-resorufin ether in PBS suggest that it undergoes two electron transfer on a GCE (Fig. 1) The separation between the oxidation and reduction peaks for scan rates at 100–600 mV s^{-1} gave an average value of 37 mV. This is somewhat higher than theoretically predicted (29.5) mV, but still much lower than the theoretical value for 1 electron transfer of 59 mV [9]. A plot of the square root of scan rate vs. peak anodic current was linear, suggesting diffusion controlled mass transport of methoxy-resorufin ether to the electrode. Chronoamperometry measurements were performed in order to estimate a value of the diffusion constant D of MROD. The current time response was re-plotted as $1 / i^2$ vs. time, but with all data in the first two seconds after the pulse was disregarded, as this current contains a significant contribution from the capacitive charging of the electrode. A linear fit of the data,

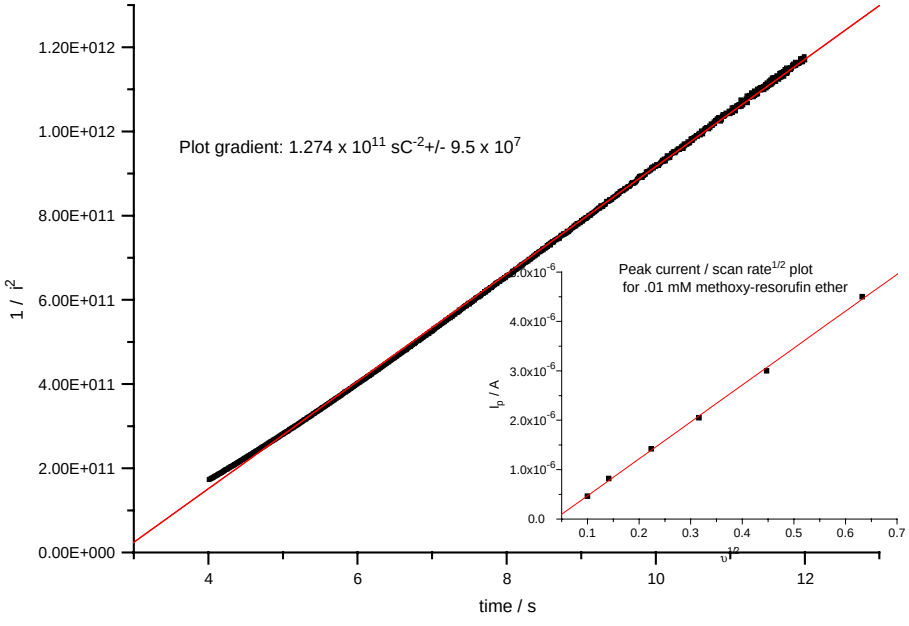

Fig. 2. Cotrell plot ($1/i^2$ vs. t) for 0.1 mmol dm^{-3} methoxy-resorufin ether for determination of diffusion coefficient. Inset is plot showing linear relationship between square root of sweep rate and peak anodic current.

shown in Fig. 2 revealed a diffusion coefficient for methoxy-resorufin ether in PBS to be 1.3×10^{-9} $m^2 s^{-1}$.

3.2. Electrochemical study of methoxy-resorufin ether O-demethylation by CYP6G1

For this study it was necessary to have a low concentration of methoxy-resorufin ether, in order that substrate depletion might be observed. The concentration was calculated and adjusted from the results of the Hikone-R fluorescence study (see Section 3.3) made in the presence of NADPH. The problem with working with low concentrations of methoxy-resorufin ether is that the charging current of the electrode obscures the Faradaic electrochemical response of methoxy-resorufin ether itself. For this reason it was necessary to use differential pulse voltammetry (DPV), which effectively subtracts the charging current contribution to the total measured current allowing voltammetric resolution of low concentration electroactive species.

Fig. 3 shows the change in cathodic current of methoxy-resorufin ether as it is metabolised by the Hikone-R (resistant) and Canton-S (susceptible) microsomes. A clear difference in rate of substrate metabolism was observed. Further electrochemical measurements were performed to study methoxy-resorufin ether metabolism by the protein microsomes, in the presence and absence of both the co-factor NADPH and CO. Fig. 4 shows that methoxy-resorufin ether metabolism (*O*-demethylation) is both NADPH dependent and inhibited by CO.

The reason for the increase in cathodic current observed at around 20 min is not clear, but may correspond to the enzymatic metabolism of methoxy-resorufin ether being more efficient when methoxy-resorufin ether has been first electrochemically reduced. In this case an enhanced cathodic current would be expected, via an *EC* type reaction

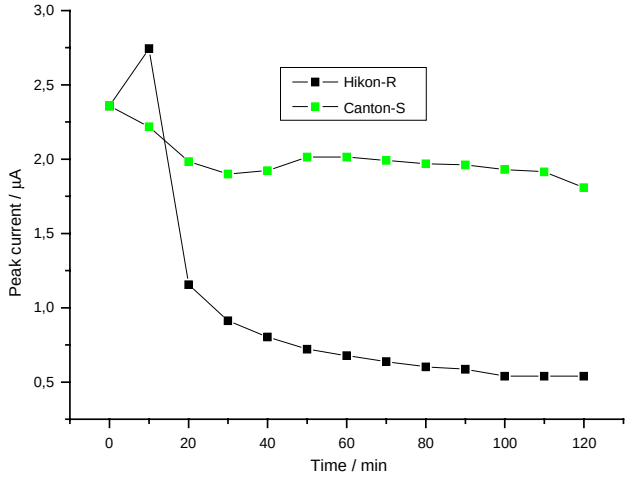

Fig. 3. Electrochemical determination of substrate (methoxy-resorufin ether) concentration measured as a function of time for microsomes from resistant (Hikone-R) and susceptible (Canton-S) strains. Note the increased rate of substrate metabolism for resistant strain. NADPH was present in both experiments.

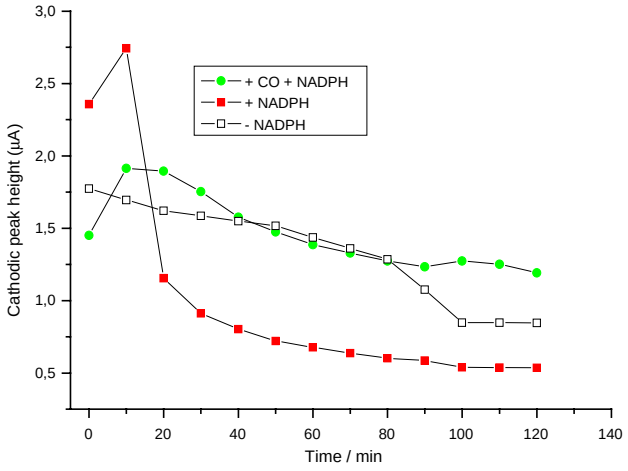

Fig. 4. Electrochemical determination of NADPH dependence and CO inhibition of methoxy-resorufin ether metabolism by resistant (Hikone-R) microsomes.

mechanism, the enzymatic metabolism enhancing the electrochemical reduction. The overall decrease in peak current with time for the two control systems is attributed to electrode fouling by the protein microsomes.

3.3. Fluorescence assay

To further validate the electrochemical measurements a fluorometric assay was used to measure the concentration of the fluorophore resorufin, the product of CYP6G1 metabolism of methoxy-resorufin ether. Although fluorescence is inherently more sensitive than electrochemistry, this technique measures the metabolite concentration rather than the actual substrate concentration.

Fig. 5 shows the change in fluorescence for microsomes from insecticide resistant (Hikone-R) and susceptible (Can-

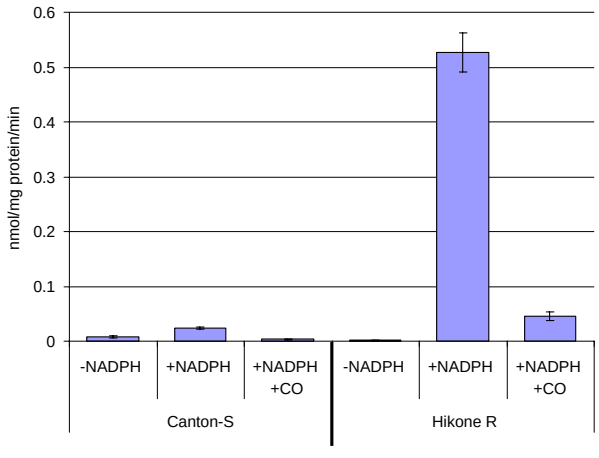

Fig. 5. Fluorescence determination of resorufin production by microsomes from susceptible (Canton-S) and resistant (Hikone-R) flies, showing number of moles of metabolic product being produced per minute/mg of protein. Metabolism of the methoxy-resorufin ether substrate is both NADPH dependent and CO inhibitable, and substrate metabolism is increased in resistant preparations as they contain more CYP6G1 enzyme.

ton-S) flies. In order to determine the cofactor dependence of the fluorometric response, two control measurements were carried out. First measurements were made in the absence of NADPH and secondly measurements were made in the presence NADPH and the *P*450 specific inhibitor CO. The resistant microsomes, which contain more CYP6G1 showed greater rates of substrate metabolism than those from susceptible flies under these conditions. These results confirm earlier gene analysis of insecticide resistant *Drosophila*, a single gene, *Cyp6g1,* is over transcribed. [2]

4. Conclusions

This paper presents the first electrochemical measurements of the important insecticidal cytochrome *P*450 CYP6G1. The extraction of protein microsomes containing CYP6G1 from two strains of *Drosophila* was described. The electrochemical measurements of the methoxy-resorufin ether substrate allowed the effect of over transcription of a single gene over the presence or absence of NADPH and the effect of carbon monoxide to be investigated. This proved to be an effective way of directly following substrate metabolism and compared well with what is known about the genetic differences in the two strains of *Drosophila* from which the protein microsomes were extracted. The results correlated with fluorescence measurements of the substrate metabolic product. Similar electrochemical approaches will therefore be useful in the measurement of other xenobiotic metabolising *P*450s, where electrochemically active drug related substrates are available or can be designed, for example, *P*450 mediated metabolism of the well known, but liver toxic pain killer, paracetamol (*N*-acetyl-*p*-aminophenol being electrochemically active).

Finally, the redox active properties of methoxy-resorufin ether, and the possibility of conjugating the sodium salt of resorufin to biologically important molecules such as DNA or proteins, suggest that it could be a useful ‘dual read out’ probe, with both fluorometric activity (though less strong than the free resorufin) and electrochemical response.

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