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N-(2-Ferrocene-ethyl)maleimide: a new electroactive sulphydryl-specific reagent for cysteine-containing peptides and proteins

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Abstract We report the synthesis and application of a specific electroactive label, *N*-(2-ferrocene-ethyl)maleimide, which provides new redox properties to organic compounds and proteins possessing sulphydryl groups. Its reaction conditions with the cysteine-containing peptide, glutathione, and a terminal mono-oxygenase enzyme, cytochrome $P450_{cam}$ are presented. The labelled peptide and enzyme acquired reversible electrochemical properties due to the attached ferrocene moiety.

Key words: Electroactive label; Sulfhydryl-specific reagent; Cytochrome P-450; Glutathione; Electrochemistry

1. Introduction

The sulphydryl groups of enzymes and peptides possess a high degree of reactivity with a variety of agents. This distinctive property permits the design of selective reagents that react rapidly and stoichiometrically with proteins and which may be employed under relatively mild conditions. N-substituted maleimides contain an activated double bound and have been shown to be particularly useful for labelling proteins since they only react with the most reactive sulphydryl groups to form stable thioethers. Based on the maleimide moiety, we designed and synthesised a ferrocene-containing reagent in order to introduce an electroactive label at chosen sites into proteins. To check its reactivity towards SH groups, we have modified the tripeptide, glutathione, and used the resultant compound as a control in our electrochemical experiments when comparing results with a labelled enzyme. The selectivity of the reagent towards cysteine residues was tested on the enzyme β-lactamase I from *Bacillus cereus*, which itself does not contain this amino acid in its sequence.

The naturally occurring cytochrome $P450_{cam}$ from *Pseudonomas putida*, a mono-oxygenase that catalyses the formation of 5-exo-hydroxycamphor from camphor [1], was selected for modification with the electroactive ferrocene label. This enzyme contains 8 cysteine residues [2,3]. As cytochrome $P450_{cam}$ is a redox enzyme itself, we predicted that the coupled label would add additional redox properties to the molecule.

In this paper we give details of the synthesis of the ferrocene label and on conditions for the chemical modification of glutathione and the enzyme. We also provide an account of the electrochemical behaviour of the labelled peptide and protein and a comparison of the activity of the modified cytochrome $P450_{cam}$ with that of the unmodified enzyme is given.

2. Materials and methods

2.1. Materials

Chemicals and solvents used were reagent grade and used without further purification unless otherwise noted. Water for all solutions was purified using a Milli-Q water purification system (Millipore).

β-Lactamase I from *B. cereus* was purchased from Sigma Chemical Co.

Putidaredoxin and putidaredoxin reductase were prepared according to literature methods [4–6].

Wild-type cytochrome $P450_{cam}$ was overexpressed in the *E. coli* strain JM 109 containing the gene of the wild-type cytochrome $P450_{cam}$ and the bacterial growth and protein purification were carried out by slight modification of published procedures [7,8].

2.2. Methods

DC cyclic voltammetry experiments were performed at room temperature in a two-compartment glass cell with a working volume of 0.5 ml. The working compartment accommodated the platinum gauze counter electrode in addition to the edged plane pyrrolytic graphite working electrode. A saturated calomel electrode (SCE) was used as a reference in a sidearm which connected to the working compartment via a Luggin capillary. All potentials are referred to the SCE. An Autolab R potentiostat (Eco Chemie B.V.) was used to record and control the potential of the working electrode. All measurements were made in 40 mM potassium phosphate buffer pH 7.4 (in the case of cytochrome $P450_{cam}$ this contained 1 mM camphor).

2.3. Synthesis of N-(2-ferrocene-ethyl) maleimide (Fc-Mi)

2.3.1. N-(2-Ferrocene-ethyl)maleamic acid. 460 mg (2 mmol) 2-ferrocene-ethylamine (synthesised by literature methods [9]) was dissolved in 10 ml ice-cold dry tetrahydrofuran and mixed with 220 mg (2.4 mmol) maleic anhydride, also dissolved in ice-cold tetrahydrofuran. The mixture was allowed to react for 1 h by stirring at 4°C, then its volume concentrated on a rotary evaporator to 5 ml. The crude reaction mixture was chromatographed on a silica gel column, previously equilibrated with 10% methanol containing ethylacetate. The clean compound eluted with a retention factor of 0.5. The corresponding fractions were collected, concentrated and trituated with ether to yield 480 mg of a yellow solid.

Melting point: 128–130°C. Elemental analysis: Expected % of C: 58.72; H: 5.20; N: 4.20; Obtained % of C: 58.96; H: 5.20; N: 4.18.

2.3.2. N-(2-Ferrocene-ethyl)maleimide. 400 mg (1.2 mmol) *N*-(2-Ferrocene-ethyl)maleamic acid was added to a solution of 4 ml acetic anhydride containing 15% ammonium acetate. The resulting suspension was heated to 100°C for 10 min with stirring. The reaction mixture was then poured into 30 ml cold water, 20 ml ethyl acetate was added and the reaction product extracted twice more into ethyl acetate. The organic phases were collected, washed 3 times with 5% $NaHCO_3$, twice with brine and dried over magnesium sulphate. The solution was concentrated and applied onto a silica column. The product was eluted with ethyl acetate with a retention factor of 0.9. The pure fractions were pooled and the solvent removed to give 220 mg yellow solids. Melting point 98–100°C.

The compound was characterised by the following spectral data: IR ν_{max} ($CHCl_3$) (cm^{-1}): 1780 (w), 1710 (s) (C=O maleimide); mass spectrometry gave a molecular mass of 309 Da. Elemental analysis:

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Expected % of C: 62.14; H: 4.85; N: 4.52; Obtained % of C: 62.70; H: 4.35; N: 4.42.

2.4. Modification of proteins and glutathione with Fc-Mi

The cys-containing peptide glutathione was used as a model to determine the conditions for the modification of cytochrome $P450_{cam}$ with Fc-Mi. The disappearance of its SH group, followed spectrophotometrically using the sulphydryl reagent 5,5'-dithiobis(2-nitrobenzoic acid) [10], indicated the end of the reaction.

Stock solutions: cytochrome $P450_{cam}$ (20 μM in 40 mM potassium phosphate pH 7.4, 1 mM camphor), β-lactamase I (20 μM in 40 mM potassium phosphate pH 7.4), Fc-Mi (2 mM in ethanol).

In a typical reaction, 2 ml of cytochrome $P450_{cam}$ and 14 μl of Fc-Mi (molar ratio of 1:1.2, enzyme:modifier) were mixed and left at 4°C for 15 min. At the end the protein mixture was passed through a PD10 Pharmacia column to remove excess reagent, purified by FPLC, and used for electrochemical and enzyme activity measurements. In the case of β-lactamase I, 2 ml of protein solution was treated with 100 μl of Fc-Mi (molar ratio of 1:10 enzyme:modifier) and left to react for 10 h at 4°C. After removing excess of reagent it was further purified on a Mono S (Pharmacia) column.

The modification of glutathione was as follows: to 5 ml 0.1 mM aqueous peptide solution 300 μl of Fc-Mi stock solution was added, the excess ferrocene reagent removed by ethyl acetate extraction, the aqueous phase was diluted to 10 ml with 100 mM potassium phosphate buffer pH 7.4 and used directly for electrochemical measurements.

2.5. Enzyme concentration and activity measurements

Enzyme concentrations were determined spectrophotometrically, using a Hewlett Packard 8451A Diode Array spectrophotometer. Absorption measurements were taken at 391 nm for the camphor bound cytochrome $P450_{cam}$ and concentrations calculated from its extinction coefficient of 102 mM^{-1} cm^{-1} [11]. The concentration of β-lactamase I was determined at 280 nm utilizing a molar extinction of 231 mM^{-1} cm^{-1} [12].

2.6. Cytochrome $P450_{cam}$ activity assay [11]

In order to compare the effect of ferrocene modification on the native enzyme the consumption of NADH was measured in the fully reconstructed system and monitored spectrophotometrically at 340 nm. Conditions were such that the rate-limiting factor for NADH oxidation was the enzyme concentration: 16 μM putidaredoxin, 0.5 μM putidaredoxin reductase, 0.05 μM cyt$P450_{cam}$ and 250 μM NADH.

3. Results

The newly synthesized electroactive label Fc-Mi remained stable over a year without decomposition at 4°C as shown by its spectral data and repeated elemental analysis of the compound. Glutathione was used to study the reaction conditions

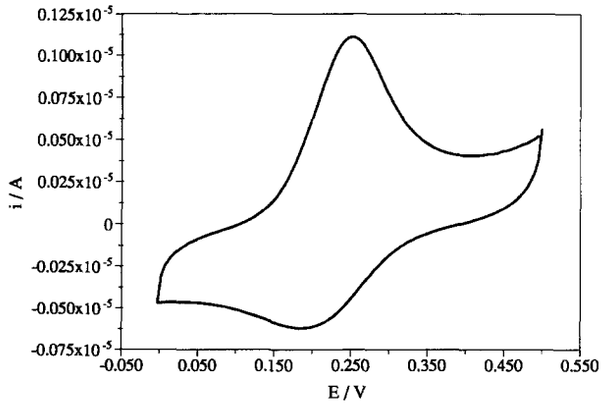

Fig. 1. Cyclic voltammogram of 50 μM Fe-Mi taken in 40 mM potassium phosphate buffer (pH 7.4) at 50 mV s^{-1} scan rate.

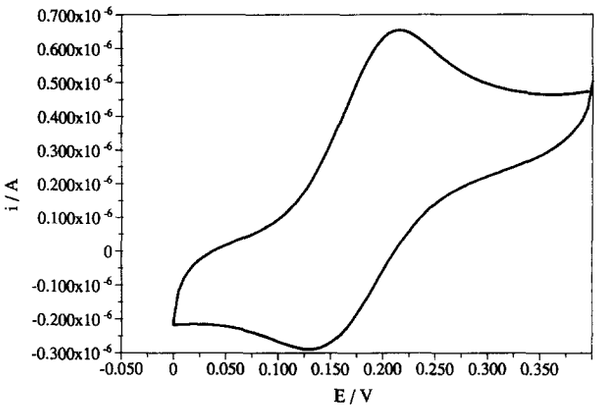

Fig. 2. Cyclic voltammogram of ferrocene-glutathione conjugate (40 μM) in potassium phosphate buffer (pH 7.4) at a scan rate of 50 mV s^{-1}.

and reactivity of the Fc-Mi. The thioether formation was fast, going to completion in less than 20 min (as assessed by the Ellman method [10]) and the excess of reagent needed to complete the reaction was not more than 20%. This reaction can be performed under conditions where enzymes remain active (pH, temperature, buffer). Based on these observations, freshly purified wild-type cytochrome $P450_{cam}$ was treated with Fc-Mi. We found that short reaction time was essential to obtain active Fc-modified enzyme, with 78% of the activity remaining after 15 min. If the reaction time was longer, then enzyme activity was gradually lost, suggesting a biphasic kinetic behaviour.

Fig. 1 shows the cyclic voltammogram (CV) of the Fc-Mi modifier alone, and its change in shape and redox potential when coupled to the glutathione is presented on Fig. 2. The asymmetric CV becomes more symmetric and its redox potential shifts from 230 to 170 mV.

Electrochemical measurements of the Fc-modified cytochrome $P450_{cam}$ are presented in Fig. 3. The CV of the compound clearly shows the incorporation of ferrocene into the protein as seen by the reversible oxidation-reduction peak at 260 mV with a peak separation of 68 mV, along with the redox couple of cytochrome $P450_{cam}$ at −380 mV [13]. In contrast, the Fc-Mi treated β-lactamase I did not exhibit any electrochemistry as its CV (Fig. 4) demonstrates.

4. Discussion

The conjugation reactions of Fc-Mi with glutathione and cytochrome $P450_{cam}$ were fast and selective under conditions where the enzyme remained active. The relatively short reaction time limit required for the cytochrome $P450_{cam}$ to maintain activity was a consequence of a secondary inhibitory effect of the label on this particular enzyme. (This inhibitory effect is being investigated further and the results will be published elsewhere.) However, in the first fast phase, when 20% excess of modifier was used, only about 20% activity is lost following the incorporation of ferrocene label after reacting with Fc-Mi for only 15 min.

Electrochemical results with the labelled glutathione and enzymes proved that the Fc-Mi reacted selectively with their sulphydryl groups to form electroactive derivatives. The selectivity of the reagent towards cys residues was proved by the fact that when β-lactamase I was treated with a large excess of

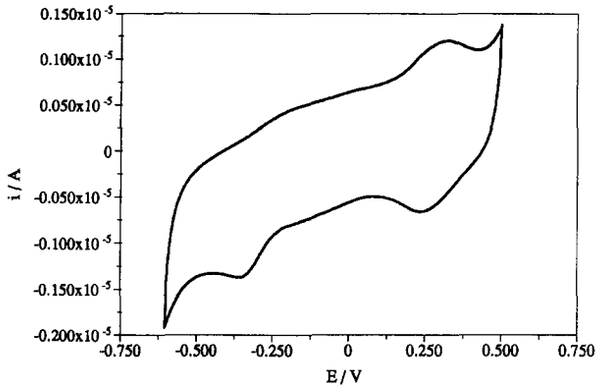

Fig. 3. Cyclic voltammogram of ferrocene-labelled Cyt $P450_{cam}$ taken in a deaerated 40 mM phosphate buffer, pH 7.4, containing 1 mM camphor. The concentration of the modified enzyme was 10 μM. Scan rate: 50 mV s^{-1}.

Fc-Mi no incorporation of ferrocene was detected as its CV (Fig. 4) gave only a plain background current. The rather asymmetric CV of the modifier Fc-Mi (Fig. 1) becomes symmetric both upon reacting with glutathione (Fig. 2) and with the enzyme (Fig. 3). This finding suggests that the Fc-Mi itself may interact with the electrode but, after reacting with the cysteine residues, shows normal reversible electrochemistry. Thus, upon reaction with cysteine residues the Fc-Mi behaves differently towards the electrode probably because its chemical reactivity was lost after its covalent attachement to the peptide or the enzyme.

As the peptide and the protein became labelled with ferrocene, each acquired a reversible redox couple characteristic of the ferrocene moiety. Because the modifier was used in just 20% excess, it was likely that only the most reactive cysteine residue in the enzyme was modified. Cyt $P450_{cam}$ possesses a well defined positive patch on its surface around the proximal heme site, which is the electron transfer site for its natural

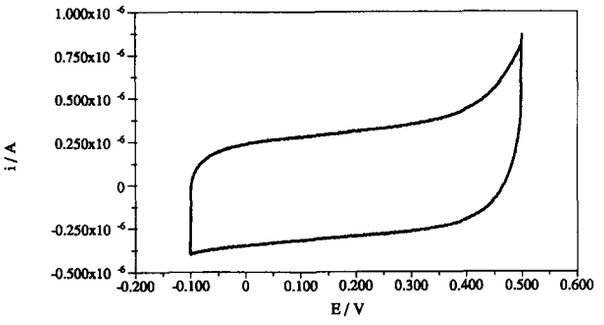

Fig. 4. Cyclic voltammogram of ferrocene maleimide treated β-lactamase (10 μM in 40 mM potassium phosphate buffer, pH 7.4). Scan rate: 50 mV s^{-1}.

redox partner: putidaredoxin [14,15]. This charge constellation plays role in orienting the enzyme towards the negatively charged electrode surface. Since one of the cysteine residues of the cyt $P450_{cam}$ is next to this docking site and readily accessible on the surface of the enzyme as shown by X-ray crystallography [16], it is probable that it is this cysteine residue that reacts with the ferrocene label and is responsible for the reversible electrochemistry obtained at 260 mV.

The main objective of this work was to demonstrate the facile and selective introduction of a novel electroactive SH-reagent, FcMi, into cysteine-containing peptides and proteins. Its excellent redox properties allow the electrochemical study of proteins, enzymes or peptides even if they are originally electroinactive. For instance site specific introduction of the group into proteins could lead to novel electrochemical sensors. In other cases, for example, surface cysteines in transport proteins modified with FcMi, could give valuable information about many structural and functional aspects of the system analysed. Given more systematic investigations, this reagent could be developed into a new tool to study simple or complex biological systems electrochemically.

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