Site-specific introduction of an electroactive label into a non-electroactive enzyme (β-lactamase I)

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Abstract A cysteine residue was introduced close to the active site of β-lactamase I by site-directed mutagenesis to replace tyrosine-105 and was subsequently modified with an electroactive SH-specific reagent, *N*-(2-ferrocene-ethyl)maleimide. The resulting modified enzyme became electroactive, showing good quasi-reversible electrochemistry which was characteristic of the attached ferrocene moiety while retaining its specific enzymatic activity. In the presence of a suicide substrate, 6β-iodopenicillanic acid, the redox potential shifted +20 mV suggesting that the label was sensitive to changes in the active site of the enzyme.

Key words: Electroactive label; Sulphydryl-specific reagent; β-Lactamase I from *Bacillus cereus*; 6β-Iodopenicillanic acid; Electrochemistry

1. Introduction

Over the past decade new methods have been developed that allow the electrochemical study of redox proteins and enzymes [1,2]. To extend the range of enzymes which could be analysed electrochemically we have investigated the possibility of introducing redox-active groups into enzymes site-specifically and thus open them up to study by electrochemical methods. To achieve the site-specific placement of a redox-active group, one of the options is to introduce a thiol group into proteins by a single cysteine substitution at the desired position. Out of several nucleophilic groups found in proteins the sulphydryl group of cysteine is by far the most reactive. If the enzyme to be modified does not have other reactive cysteines, by replacing an amino acid residue by cysteine it becomes possible to introduce the electroactive label site-specifically into the protein.

Recently we reported the synthesis of a ferrocene-containing sulphydryl reagent: *N*-(2-ferrocene-ethyl)maleimide (Fc-Mi) [3]. This label has now been used in our attempt to develop a general method to arrive at potentially new electrochemical biosensors.

As a model, we have studied the possibility of converting the redox-inactive enzyme β-lactamase I from *Bacillus cereus* into an electroactive enzyme. β-Lactamase I is a clinically important serine protease which lacks cysteine residues [4,5]. The 3-dimensional structure of this enzyme was provided by D. Stuart (personal communication). With the use of molecular graphics we have chosen possible sites that would allow the easy introduction of the electroactive label without clashes thereby minimising disturbance to the protein's structure. In this way we hoped to achieve the aim of introducing an electroactive group that would be sensitive to changes in the environment of the active site. Cysteine residues were introduced into strategically important positions and the mutant enzymes so obtained were further modified with Fc-Mi.

Changes at the labelled enzyme's active site were studied by using inhibition of the modified β-lactamase I [6]. 6β-Iodopenicillanic acid (6β-IPA) is a suicide substrate of β-lactamase I [7,8] which upon enzyme catalysis becomes irreversibly captured by the enzyme and so causing its inactivation.

Herein we give details on the preparation and expression of three mutant β-lactamases and the conditions for their chemical modification with Fc-Mi. The electrochemical and kinetic behaviours of the wild-type enzyme and one of the successfully modified enzyme-mutant in the presence of a substrate/or an inhibitor are compared and discussed.

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). Nitrocefin was a gift from Dr. J. Errington, Sir William Dunn School of Pathology Oxford, 6β-iodopenicillanic acid was a gift from Pfizer Ltd., Kent.

β-Lactamase single mutants, where either Tyr^{105}, Ser^{129} or Trp^{210} were replaced by cysteine, were constructed in the phagemid vector $pKS_{cat}\beta l$ (obtained from T. Leung, Sir William Dunn School of Pathology Oxford) using the uracil template protocol [9] and expressed in *Bacillus subtilis* [10] with the bacteriophage vector pSG703. In this expression system the enzyme is secreted into the medium after heat shock and 6 h growth. The purification of the mutant enzymes was achieved by adsorption to, and elution from, Celite, followed by ion exchange chromatography on CM Sephadex G50 (Pharmacia) column using 50 mM Tris-HCl buffer (pH 7) with a gradient (final concn.: 1 M KCl). All buffers used were degassed and contained 10 mM DTT and 1 mM EDTA to prevent auotooxidation of the sulphydryl group of the cysteine. Fractions containing β-lactamase activity were pooled, concentrated using Amicon 10 ultrafiltration membrane and desalted by exchanging the buffer into the initial low salt buffer. This protein solution was passed through a PD 10 (Pharmacia) column just before treating it with Fc-Mi.

2.2. Methods

Direct current (DC) cyclic voltammetry and differential pulse voltammetry experiments were carried out 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 side-arm which connected to the working compartment via a Luggin capillary. All potentials are referred to the SCE. An AUTOLAB R potentiostat by Eco Chemie BV was used to record and control the potential of the working electrode. All measurements were made in 50 mM Tris-HCl/200 mM KCl buffer (pH 7.0).

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2.3. Modification of enzymes with Fc-Mi

Stock solutions: Fc-Mi (20 mM in ethanol); β-lactamase I (20 μM in 50 mM Tris-HCl buffer (pH 7.0).

Two millilitres of protein solution and 24 μl of Fc-Mi (12-fold excess of label) were mixed and left to react at 20°C for 6 h in the dark. At the end the protein mixture was passed through a PD10 (Pharmacia) column to remove excess of reagent, purified by FPLC, and used for electrochemical and enzyme activity measurements.

2.4. Enzyme concentration's measurements

Enzyme concentrations were determined spectrophotometrically, using a Hewlett Packard 8451 A Diode Array spectrophotometer. The concentration of β-lactamase mutants were determined at 280 nm using a molar extinction of 231 mM^{-1} cm^{-1} [11].

2.5. β-lactamase activity assay

Nitrocefin, as a coloured substrate for penicillinase, was used as a qualitative test to detect β-lactamase activity during purification steps. Quantitative assays with this substrate at saturated substrate concentrations were also set up [12,13] to compare activities of the wild-type, mutant and ferrocene-modified β-lactamases. Substrate hydrolysis was monitored spectrophotometrically at 500 nm.

2.6. Inactivation of β-lactamase I and its Fc-labelled mutant Y105C with the suicide substrate 6β-IPA

Stock solution of 6β-IPA: 2.4 mM in distilled water.

To a 10 μM enzyme solution in 50 mM sodium phosphate, 0.5 M NaCl buffer (pH 7) 6β-IPA was added in 1–10-fold excess of inhibitor to enzyme concentrations. The mixture was incubated for 10 min at 30°C and the suicide reaction was monitored by following the formation of dihydrothiazine at 326 nm (ε: 12 500 M^{-1} cm^{-1}; [14]). The ratio of dihydrothiazine/enzyme concentrations were calculated and used as a measure for inhibition efficiency [7].

3. Results

Freshly purified β-lactamase I mutants Y105C, S129C and W210C were treated in an identical fashion with Fc-Mi. However, after purification and iron content analysis, only one mutant proved to be modified with Fc-Mi. This was the Y105C mutant. The relative activities of the mutant enzymes and that of the modified one were determined and compared to the wild-type β-lactamase I. The activities of the mutant proteins relative to the wild-type were 95% for Y105C, 90% for S129C, 92% for W210C while the Fc-modified enzyme showed somewhat reduced activity (30% of the native). In all cases the margin of error was ± 10%.

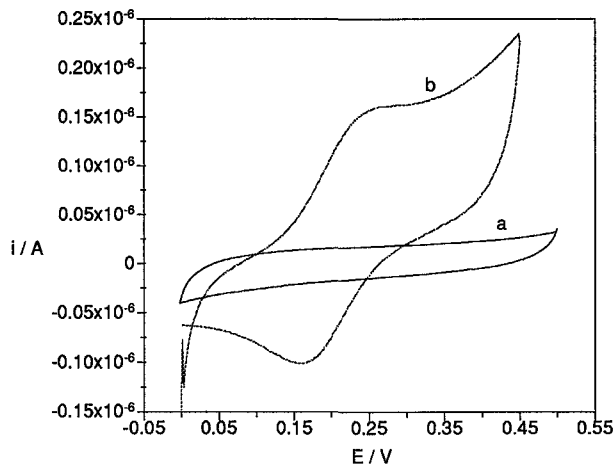

Fig. 1. Cyclic voltammograms of the wild-type β-lactamase (a) and the ferrocene-modified Y105C mutant (b) were taken in 50 mM Tris-HCl buffer (pH 7). Enzyme concentrations were 10 μM in each case. Scan rate: 20 mV s^{-1}.

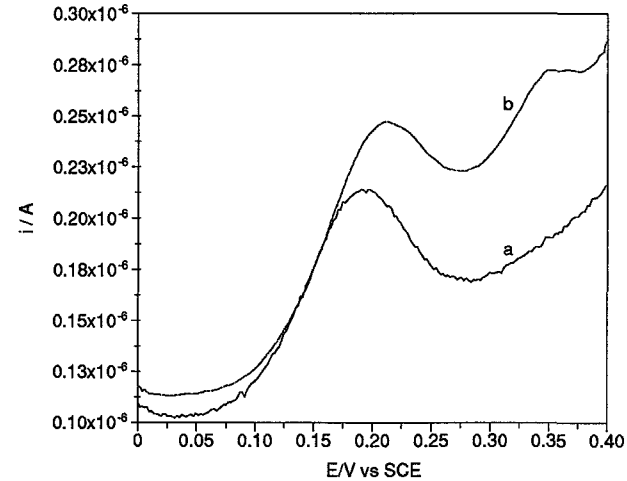

Fig. 2. Differential pulse voltammograms of the ferrocene-labelled β-lactamase mutant (10 μM in 50 mM Tris-HCl buffer, pH 7) in the absence (a) and in the presence of 60 μM 6β-IPA (b). Scan rate: 20 mV s^{-1}.

The efficiency of the suicide substrate was tested with the wild-type β-lactamase I and Fc-Mi modified β-lactamase mutant Y105C. It was found that a 3-fold excess of 6β-IPA completely inhibited enzymatic activity of the wild-type β-lactamase I in 5 min while a 6-fold excess of the inhibitor was needed to inactivate the modified enzyme within the same time.

The DC cyclic voltammogram of the unmodified and the Fc-Mi treated β-lactamase Y105C is shown in Fig. 1. While the unmodified enzyme remained electrochemically silent in the range of 0–500 mV (a), the ferrocene-modified β-lactamase Y105C shows redox activity, confirming the incorporation of the ferrocene label into the protein (b). Its DC cyclic voltammogram is consistent with the electrochemically reversible behaviour of a ferrocene/ferricinium ion one-electron redox couple ($E_{1/2}$ = 193 mV, ΔE_p = 63 mV, $i_{pa}/v^{1/2}$ = constant).

Differential pulse voltammetry experiments were carried out with the ferrocene-labelled β-lactamase I in the absence (Fig. 2a) and in the presence of 6β-IPA (Fig. 2b). The redox potential of the ferrocene-modified β-lactamase shifted from 193 to 214 mV upon addition of its suicide substrate. Here an additional oxidation peak was obtained at 360 mV.

4. Discussion

The fact that out of the three mutants expressed, we could only introduce the ferrocene label into one mutant (Y105C), might imply that, in the other two cases, the chosen sites, where the cysteine residue was inserted, were not sufficiently close to the protein's surface to react with the reagent. The relatively low specific activity obtained for the modified enzyme is probably due to the fact that the site where the label was introduced is close to its active site entrance and so the bulky ferrocene molecule perturbs the free access of the substrate into the catalytic pocket. This observation is consistent with the results obtained with the suicide substrate's efficiency test, where double amount of 6β-IPA was needed to inhibit completely the ferrocene-modified enzyme activity, compared with the wild-type enzyme.

DC cyclic voltammetry experiments with the wild-type and

the redox-labelled β-lactamase mutant Y105C (Fig. 1) demonstrates that the Fc-Mi covalently reacted with its single cysteine residue providing the latter with quasi-reversible redox properties due to the attached ferrocene moiety. The selectivity of the label towards cysteine residues was earlier demonstrated [3], and the fact that the other cysteine containing enzyme mutants (S129C and W210C) did not became electroactive upon Fc-Mi reaction further suggests that the chosen sites were not readily available for chemical reaction.

Differential pulse voltammetric studies revealed a fundamental shift in the redox potentials of the ferrocene-labelled β-lactamase Y105C mutant upon the addition of its suicide substrate: 6β-IPA (Fig. 2a,b). The presence of the covalently captured substrate analogue in the enzyme's active site had a direct electronic effect on the ferrocene moiety by increasing its redox potential. This result indicates that the label used was placed successfully close to the enzyme's catalytic centre and in a position that became intimately sensitive to changes in the active site. The additional oxidation peak attained at 360 mV in the presence of 6β-IPA was due to the formation of a redox active species, dihydrothiazine, upon substrate turnover.

The results presented in this paper not only demonstrate the feasibility of converting redox-inactive enzymes redox-active but most significantly, as the introduced electroactive label became sensitive to changes in the enzyme's active site, gives the potential to develop novel electrochemical biosensors based on the site-specifically placed ferrocene label.

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