

Effect of Immobilization on the Penicillin Binding and Reactivity Properties of DD-peptidase from *Streptomyces* R61

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An affinity gel matrix containing an enzyme (DD-peptidase) with specific β -lactam binding properties was characterized with respect to its binding and reactivity behavior with penicillin. The data show that immobilization of DDP by reaction with the enzymes susceptible amino groups resulted in changes in catalytic activity on a tripeptide substrate, penicillin binding efficiency and pH stability of drug binding. Properties unaffected by immobilization were the drug-enzyme complex stability, binding reaction mechanism, drug selectivity and method of complex desorption. The affinity of DDP for penicillin-G was investigated by surface plasmon resonance. These characteristics were compared with those of the soluble enzyme. Conditions for elution of the bound drug were determined and a method for immobilizing *Streptomyces* DDP by which its binding site structure is sustained was also evaluated.

Keywords: affinity chromatography; carboxypeptidase; DD-peptidase; immobilization; β -lactam; penicillin; penicillin-binding protein; surface plasmon resonance

Introduction

The DD-peptidases are membrane-bound penicillin-binding proteins (PBPs) which, together with the β -lactamases, form the superfamily of penicillin-recognizing proteins found in bacteria (Joris *et al.*, 1988). The two enzymes share some common physical properties: (1) they both contain an active site serine (with the exception of Zn^{2+} β -lactamases, Type B) which is the site of interaction with the β -lactam (Yocum *et al.*, 1982); (2) their kinetic mechanisms of interaction with β -lactams are similar (Christensen *et al.*, 1990); (3) their three-dimensional structures have clear similarities (Kelly *et al.*, 1986; Samraoui *et al.*, 1986); and (4) there is a high homology of sequence alignments of several conserved elements directly involved in the reaction mechanism or lining the catalytic cavity (Joris *et al.*, 1988). The most striking difference between the two enzymes, however, is the rapid hydrolysis of the bound β -lactam by the β -lactamase versus the stable complexation of the β -lactam in the active site of the DD-peptidase (suicide inactivator). For reviews, see Ghuysen (1991) and Herzberg and Moulton (1991). PBPs control bacterial cell wall biosynthesis and therefore are the bacteriocidal targets of β -lactam drugs, while the β -lactamases confer resistance to

the microorganism by inactivating the β -lactam through hydrolysis.

The extensive use of β -lactam antibiotics for veterinary purposes in food-producing animals has led to the development of numerous biological, chemical, immunological, and chromatographic methodologies for their detection (Fairbrother, 1977; Jackman *et al.*, 1990; MacNeil *et al.*, 1991; Boison, 1992; Moats, 1992; Boison *et al.*, 1994). These methods, however, do not completely satisfy the requirements of regulatory agencies with respect to rapidity, sensitivity, selectivity, affordability, simplicity, multiresidue capability and the minimal use of volatile solvents (Brown, 1993; US Department of Agriculture, 1993). Immobilized affinity ligand techniques employing non-immunological ligands for β -lactam residue capture and detection have been explored previously (Eng *et al.*, 1995). Here, we present additional results in our characterization of the affinity matrix for this class of antibiotics and discuss the effects of immobilization upon the proteins reactivity with the penicillins.

Experimental

Materials

Streptomyces R61 DD-peptidase was provided by Dr J.-M. Frere (Liege, Belgium). Penicillin-G (sodium salt) and ampicillin were obtained from Sigma, [^{14}C]penicillin-G (potassium salt, specific activity 58 mCi/mmol) from Amersham, EcoScint scintillation cocktail from National Diagnostics, acetonitrile (UV HPLC grade) from Burdick &

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Abbreviations used: DDP, DD-peptidase; iDDP, immobilized DDP; sDDP, soluble DDP; PG, penicillin-G; amp, ampicillin; DLAA, *N,N*-diacetyl-Lys-Ala-Ala; MWCO, molecular weight cut-off; NHS, *N*-hydroxysuccinimide; EDC, *N*-ethyl-*N'*-(dimethylaminopropyl)carbodiimide; UV-RP-HPLC, ultraviolet reversed-phase high-performance liquid chromatography; SPR, surface plasmon resonance; RU, response units; amp-pAb, anti-ampicillin polyclonal antibody.

Jackson, Affi-Gel 10 from Bio-Rad and *N,N*-diacetyl-Lys-Ala-Ala from Sigma. Sheep polyclonal antibody to ampicillin was obtained from The Binding Site (San Diego, CA, USA).

Methods

Immobilization of DD-peptidase (DDP). The concentration of the enzyme, which was devoid of β -lactamase activity, was determined either by its extinction coefficient at 280 nm ($E_{1\%,1\text{ cm}} = 10.0$) (Frere *et al.*, 1976) or by active site titration with penicillin-G (Frere *et al.*, 1974). In the latter method, inhibition of DDP activity with a tripeptide substrate (*N,N*-diacetyl-Lys-Ala-Ala) by penicillin was measured using the amino acid oxidase assay (Frere *et al.*, 1975). Up to 3 mg of DDP were immobilized to 500 μ L of Affi-Gel 10 in 0.1 M sodium acetate buffer (pH 4.6) following the method described previously (Eng *et al.*, 1995). The coupling efficiency was typically ca 50 per cent.

Immobilization of PG-bound DDP. In a 500 μ L volume, 0.15 μ mol of DDP was incubated with 1.04 μ mole of PG in 10 mM sodium phosphate buffer (pH 7) for 15 min at 37°C. Unbound PG was removed using a 10 kDa MWCO ultrafiltration tube (Microcon-10, Amicon). The retentate was brought up to 1.5 mL with acetate buffer (pH 4.6) and immediately applied to a microcolumn containing 500 μ L of Affi-Gel 10 previously washed and equilibrated in the acetate buffer. Coupling of the drug-bound enzyme with the gel proceeded for ca 16 h. at 4°C with end-to-end rotation. Unbound PG-DDP was collected and the column washed with the phosphate buffer. All washes were collected and combined with the unbound PG-DDP flow-through solution and assayed for protein concentration by the BCA protein assay (Pierce) and for residual peptidase activity. Unreacted NHS groups on the Affi-Gel were blocked by ethanolamine. Prior to drug binding studies using the iDDP, the column was primed by treatment with 3 per cent H₂O₂ to strip any bound PG from the coupling step.

Penicillin-binding studies. Typically, the affinity column and the drug solution were equilibrated to 25°C in 10 mM sodium phosphate buffer (pH 7). The penicillin (up to 224 μ M) was added to an equal volume of the affinity gel (112 μ M DDP) and allowed to react for 15 min. Unbound penicillin was washed off with phosphate buffer and collected for further analysis by UV-RP-HPLC. [¹⁴C]Penicillin-G was also used to measure binding. Quantitation of unbound [¹⁴C]PG was performed on a Beckman LS8000 liquid scintillation counter in which 40 μ L of the labelled sample were mixed with 4 mL of EcoScint scintillation fluid. By measuring the amount of unbound PG and knowing the total concentration of PG added to the column, the amount of bound PG can be determined.

PG binding to sDDP was measured as the extent of inhibition of tripeptide hydrolysis when the enzyme (0.2 μ M) was titrated with up to 0.39 μ M PG. In these experiments, PG was first incubated with DDP for 30 min at 25°C. The residual hydrolytic activity of the PG-DDP sample was then measured by the amino acid oxidase assay in the presence of 2 mM DLAA. Incubation of PG-inhibited DDP with DLAA was allowed to proceed for 5 min at 37°C.

Because the PG-DDP complex has a relatively short half-life (1.5 h) at 37°C, complex breakdown and enzyme reactivation can occur during the incubation step with the tripeptide substrate. In order to account for this error, the following equation was used to calculate the exact residual enzyme activity after 300 sec of incubation with the substrate (Frere *et al.*, 1974):

Corrected residual enzyme activity =

$$\frac{(S_{PG}/S_0) + [(1 - e^{-kt})/kt] - 1}{(1 - e^{-kt})/kt} \quad (1)$$

where S_{PG} = amount of hydrolyzed tripeptide substrate measured in the reaction of PG-inhibited DDP with DLAA, S_0 = amount of hydrolyzed tripeptide substrate measured in the reaction of uninhibited DDP with DLAA, t = duration of reaction between DDP with tripeptide (300 s) and k = rate constant for the breakdown of the PG-DDP complex ($1.4 \times 10^{-4} \text{ s}^{-1}$). For all PG-titrated DDP samples, the corrected residual activity values determined using Eqn (1) differed by no more than 6 per cent from uncorrected values (S_{PG}/S_0). Thus, during the term of incubation of PG-DDP with substrate (300 s), the extent of enzyme reactivation resulting from complex degradation is insignificant. Breakdown of the complex can also occur during the time span of the titration procedure, but since the titration is carried out at 25°C, the error is probably negligible. Assuming that the degree of inhibition of enzyme activity is directly related to the binding of PG in the enzyme active site, the amount of PG-complexed DDP can be determined. All binding curves were fitted to a hyperbolic curve-fitting program using SigmaPlot (Jandel Scientific).

Surface plasmon resonance analysis of ampicillin binding to immobilized DDP. The real-time measurement of the dissociation constant, K_d , for the interaction between ampicillin and immobilized DDP was made using a BIAcore biosensor system (Pharmacia Biosensor, Piscataway, NJ, USA) at 25°C. The phenomenon of SPR and its applications in kinetics, binding site analysis and concentration measurements are described in full detail elsewhere (Fagerstam *et al.*, 1992; Fagerstam and O'Shannessy, 1993). Generally, SPR occurs when light impinging on a thin metal film (i.e. gold) experiences resonant coupling to oscillating conducting electrons (plasmons) at the metal surface. This resonance is very sensitive to the refractive index of the buffer close to the sensor surface and is observed as a change in the angle of the reflected light intensity (SPR angle). Since mass determines refractive index, the change in light intensity is directly proportional to the mass associated with the surface. Resonance units (RU) express these changes in the SPR angle and are monitored with time. One RU corresponds to a change in surface concentration of ca 1 pg/mm^2 (Stenberg *et al.*, 1991).

In all immobilization experiments using the biosensor, flow rates were set at 3 μ L/min and the running buffer was HBS[10 mM HEPES (pH 7.5), 150 mM NaCl, 3.4 mM EDTA, 0.05 per cent BIAcore surfactant P20 (Pharmacia)]. Immobilization of DDP on a CM5 sensor chip (Pharmacia) was achieved by injecting 30 μ L of a 24 μ M solution diluted in 10 mM sodium acetate (pH 4.5) on to the biosensor surface pre-activated with NHS and EDC (25 μ L of a

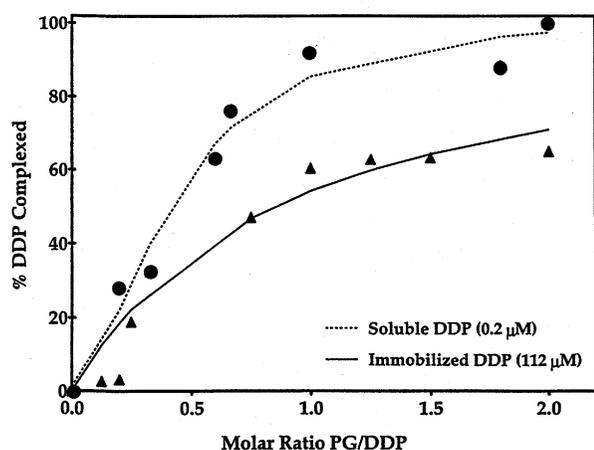


Figure 1. Titration of soluble and immobilized DDP with PG. All titration studies were conducted in 10 mM sodium phosphate buffer (pH 7) at 25°C. Binding of PG to 0.2 μM soluble DDP was determined by measuring the inhibition of DDP enzymatic activity in the presence of varying concentrations of PG. The binding of PG to 112 μM immobilized DDP was quantitated by UV-RP-HPLC. [^{14}C]PG was also used to titrate immobilized PG and similar results were obtained. Computer-aided curve fitting was performed on SigmaPlot. The K_d values determined from a hyperbolic fit of the data are 89 nM for sDDP and 20 μM for iDDP.

0.05 M NHS–0.2 M EDC mixture in distilled water). This was followed by the injection of 25 μL of 1 M ethanolamine–HCl (pH 8.5) to deactivate unreacted ester groups.

The lower molecular weight limit of detection for molecules interacting at the biosensor surface is 1 kDa. Therefore, the binding of the ampicillin molecule (349 Da) on the immobilized DDP surface cannot be analyzed directly using SPR. Detection of bound ampicillin was achieved, however, if an anti-ampicillin polyclonal antibody (amp-pAb) was used to enhance the detection signal by specifically interacting with any DDP-bound drug on the

surface (Fagerstam and O’Shannessy, 1993).

For the SPR experiments, the purified amp-pAb was diluted in HBS. Ampicillin was diluted into HBS to concentrations ranging from 1.75 to 112 mM. Each binding cycle was run at a flow rate of 3 $\mu\text{L}/\text{min}$ and consisted of the following injection steps: (1) 15 μL of the ampicillin solution; (2) 15 μL of a 2 μM (316 $\mu\text{g}/\text{mL}$) solution of amp-pAb; (3) 4 μL 100 mM HCl; (4) 21 μL of 3 per cent H_2O_2 . Between each sample pulse, the surface was washed for 100 s with HBS. Steps (3) and (4) were used to dissociate bound pAb and ampicillin, respectively. The equilibrium RU (R_{eq}) values from the sensorgrams were used to construct Scatchard plots, R_{eq} versus $R_{\text{eq}}/[\text{amp}]$ where [amp] is the concentration of ampicillin injected over the sensor surface. The binding of pAb to DDP-captured ampicillin was inhibited when pAb was pre-incubated with 10 mg/mL ampicillin for 10 min at 25°C, indicating that the change in response observed in the sensorgram after the end of the pAb injection is due to its specific interaction with surface-bound ampicillin.

[^{14}C]PG–DDP complex dissociation. The dissociation of the PG–iDDP complex was examined with either hydroxylamine or hydrogen peroxide and using [^{14}C]PG. The amount of enzyme immobilized was 53.5 nmol. An equi-volume based on the gel of either 0.8 M neutral hydroxylamine in 10 mM sodium phosphate (pH 7) or 3 per cent hydrogen peroxide was introduced into the column containing drug-bound DDP and allowed to react for 30 min at 25°C. The dissociation reaction was repeated three more times when NH_2OH was used and two more times when H_2O_2 was used. All eluates (500 μL) and wash fractions (1 mL) in the respective dissociation buffer were collected and the recovered radioactivity was determined. In NH_2OH buffer, 20 μL of each sample fraction was assayed in 4 mL of counting fluid, whereas for samples in H_2O_2 , 40 μL in 4 mL of counting fluid were assayed.

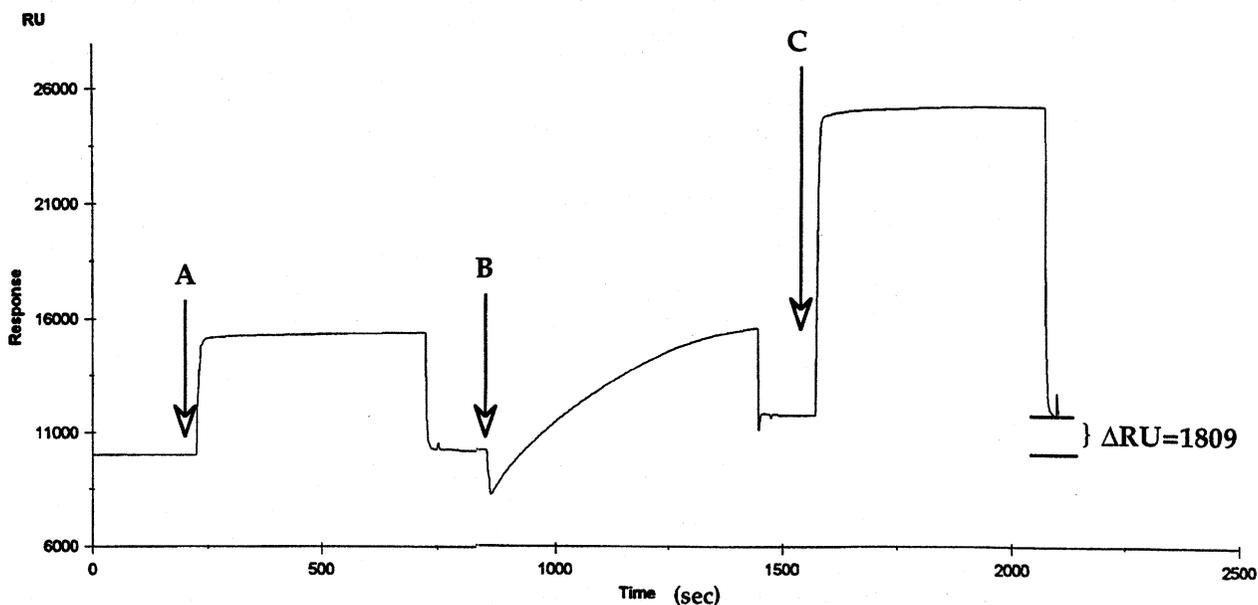


Figure 2. Sensorgram of the immobilization of DDP. All flow rates were set at 3 $\mu\text{L}/\text{min}$ and the running buffer was HBS. Pre-activation of the biosensor surface was performed by the introduction of 25 μL of a solution of 0.05 M NHS–0.2 M EDC over the surface (A). Injection of 30 μL of a solution of DDP (24 μM was prepared in 10 mM sodium acetate, pH 4.5) was next made (B) followed by 25 μL of 1 M ethanolamine–HCl (pH 8.5) (C). The ΔRU obtained is shown.

HPLC analysis. HPLC was performed on a Rainin HPXL system controlled through the Dynamax system software. The method of Moats and Malisch (1992) for penicillin detection was used. Penicillin samples were first passed through Millex HV₄ 0.45 μm filters (Millipore). All mobile phases were prepared with Type I nanopure H₂O (Barnstead), filtered through a Kontes filtration unit using Magna nylon 0.22 μm membranes (MSI) and degassed with helium. The β -lactam was isolated on a PLRP-S polymeric column (100 \AA , 5 μm , 150 \times 4.6 mm i.d., Polymer Laboratories) equilibrated in 10 per cent acetonitrile–10 mM sodium phosphate (pH 7). Elution with a linear acetonitrile–phosphate buffer gradient was applied followed by detection of the emerging β -lactam at 220 nm (Kratos Spectroflow 773). The flow rate was 1 mL/min and the sensitivity was set at 0.001 a.u.f.s. Peak integration was carried out using the Dynamax software. Calibration curves of peak area or height versus penicillin concentration were

linear up to 200 ng of penicillin based on 70 μL injections of standard solutions prepared in 10 mM sodium phosphate buffer.

Results

Titration of soluble and immobilized DDP with PG

A comparison of the PG binding behaviour was made between soluble and immobilized DDP in 10 mM sodium phosphate (pH 7) (Fig. 1). Soluble DDP (0.2 μM) was titrated with up to 0.39 μM PG and drug binding by DDP was measured as the degree of inhibition of enzyme activity as a result of its interaction with PG. This approach is valid since PG is known to bind in the active site of the enzyme. Thus, 100 per cent inhibition reflects total complexation of

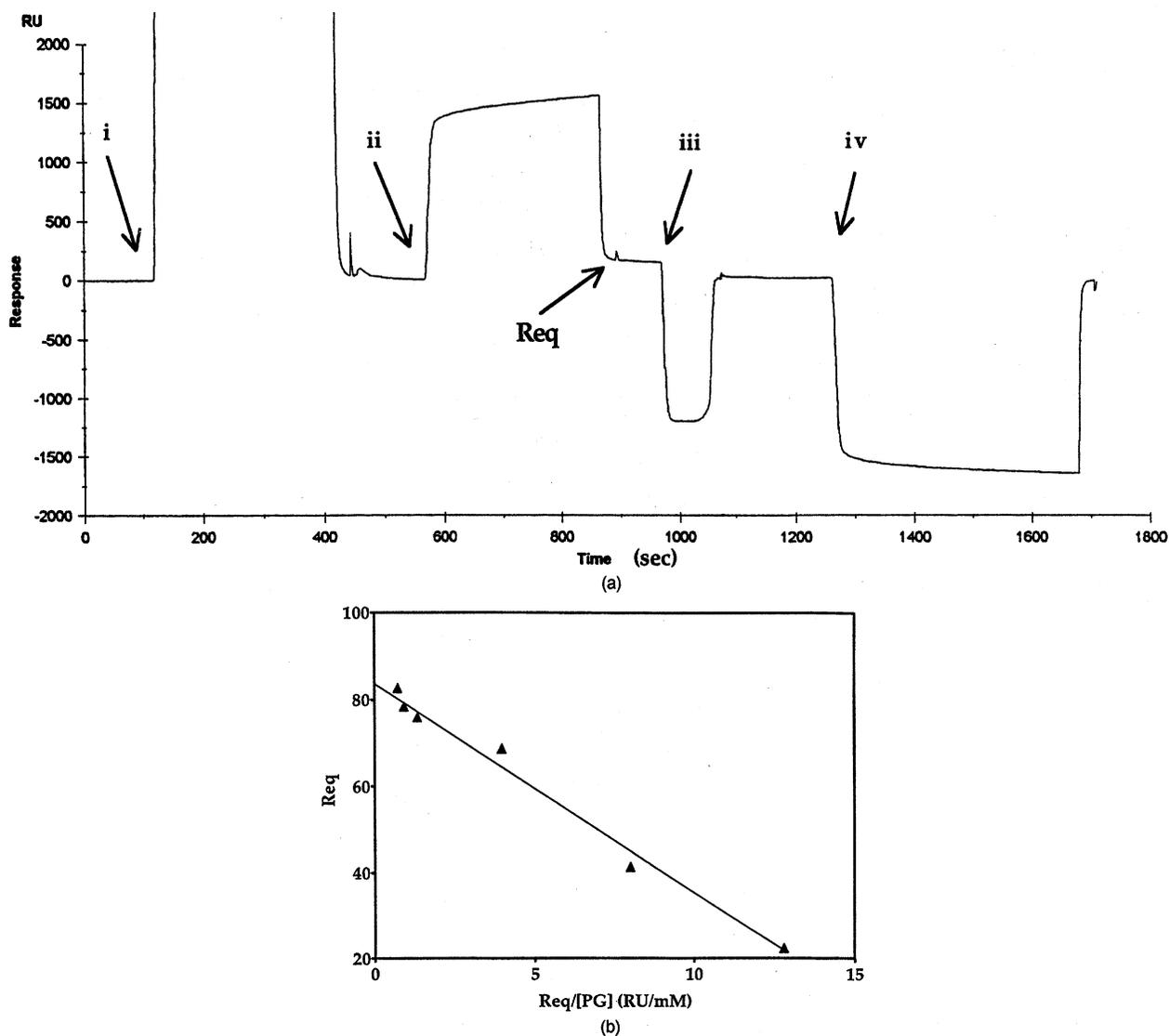


Figure 3. Evaluation of the binding of ampicillin to immobilized DDP. The sensorgram of the binding reaction is shown in (a). The flow rate was 3 $\mu\text{L}/\text{min}$ and the running buffer was HBS. All dilutions of drug (1.75–112 mM) and amp-pAb (316 $\mu\text{g}/\text{mL}$) were made in HBS. The following injection steps comprised a typical binding cycle: (i) 15 μL of ampicillin; (ii) 15 μL of 316 $\mu\text{g}/\text{mL}$ pAb; (iii) 4 μL of 100 mM HCl; (iv) 21 μL of 3% H₂O₂. R_{eq} values obtained from the sensorgram are plotted vs $R_{\text{eq}}/[\text{amp}]$ in a Scatchard analysis as depicted in (b). The correlation coefficient for the linear regression fit is 0.987.

all active DDP sites by PG. Immobilized DDP (112 μM) was titrated with up to 224 μM PG and the amount of drug not bound to the enzyme was quantitated by HPLC. Only 55 per cent of the total immobilized DDP compared with >80 per cent of soluble DDP was bound by PG when 1 mol PG/mol DDP was added. Even addition of up to 2 mol PG/mol enzyme did not significantly enhance the binding efficiency of immobilized DDP, whereas the soluble enzyme was completely complexed at this level of added PG. The K_d values determined at these low PG levels were 89 nM for sDDP and 20 μM for iDDP.

Determination of K_d for the reaction between ampicillin and immobilized DDP using plasmon resonance

The immobilization of DDP on the biosensor surface is shown as a sensorgram in Fig. 2. The coupling reaction occurs by linkage of the protein amino groups to a NHS-

Table 1. Elution of bound [^{14}C]benzylpenicillin from immobilized DDP

Treatment	Total cpm bound	Total cpm released after treatment	% cpm released	Dilution factor
NH_2OH	955	602	63	13
H_2O_2	955	1167	>100	2

A microcolumn containing 0.5 mL of gel immobilized with [^{14}C]PG-bound DDP (53.5 nmol enzyme) was reacted with either 0.8 M neutral hydroxylamine or 3% hydrogen peroxide (0.5 mL of each solution) for 30 min at 25°C. The dissociation reaction was repeated three times when NH_2OH was used and twice when H_2O_2 was used. All elutes (500 μL) and wash fractions (1 mL) in the respective dissociation buffer were collected and the recovered radioactivity was determined as described under Experimental.

activated sensor surface; thus, the enzyme in these studies should be representative of the enzyme immobilized on Affi-Gel. The amount of DDP immobilized was 1809 RU. Figure 3(A) shows a representative sensorgram for the binding of high concentrations of ampicillin to iDDP followed by the secondary binding of a polyclonal antibody (amp-pAb) to ampicillin. The sensor was unable to detect binding of the low-mass drug directly; however, addition of pAb resulted in the augmentation of the SPR signal (ΔRU). Failure to observe signal enhancement when pAb was first co-incubated with excess ampicillin in solution prior to injection demonstrates that the changes in RU reflect the specific binding of the antibody to the surface-bound drug (data not shown). The consecutive addition of 100 mM HCl and 3 per cent H_2O_2 resulted in the stepwise removal of pAb followed by ampicillin, allowing the immobilized DDP surface to be regenerated. The response levels after each cycle always returned to baseline levels after this regeneration procedure and permitted more than 20 binding cycles to be performed on the same surface. Neutral hydroxylamine (0.8 M) and NaCl (1.0 M) were not as successful in regenerating the surface (data not shown). A Scatchard plot using the R_{eq} values gave a K_d value of 4.95 mM for the interaction of ampicillin with iDDP [Fig. 3(B)].

Dissociation of [^{14}C]PG-DDP complex

Treatment of [^{14}C]PG-complexed immobilized DDP with 0.8 M hydroxylamine (pH 7) for 30 min at 25°C resulted in the release of 63 per cent of the total cpm bound to the DDP matrix (Table 1). Elution by the hydroxylamine, however, was not efficient since the recovered drug was diluted

Table 2. Comparison of ligand-binding properties of soluble and immobilized DDP

Parameter	Soluble DDP	Immobilized DDP
Catalytic activity (units) ^{a,b}	95.90 \pm 0.06	2.88 \pm 0.01
Effect of pH ^a	Unaffected by low pH	Affected by low pH
Effect of temperature ^a	No effect at 25, 37°C	No effect at 25, 37°C
Binding efficiency ^c	85%	55%
Complex stability	2.4 h ^d	>2 h ^a
Reaction mechanism ^d	Scheme 1 (low [antibiotic]) ^d Scheme 2 (high [antibiotic]) ^d	Scheme 1 (low [antibiotic]) ^a Scheme 2 (high [antibiotic]) ^a
Complex desorption ^f	NH_2OH , H_2O_2	NH_2OH , H_2O_2
Drug selectivity ^{a,g}	PG > amp > clox > 6-apa	PG > amp > clox = amox > het = ceph > 6-apa
PG binding in fortified samples ^{a,h}	No sample deproteinsation required	Sample deproteination required

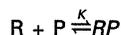
^a Results from Eng *et al.* (1995).

^b Units = μmol of D-Ala formed per minute at 37°C using DLAA as substrate.

^c Results from Fig. 1.

^d Mechanisms proposed by Nieto *et al.* (1973) and Frere *et al.* (1975):

Scheme 1: Reversible complex dissociation



Scheme 2: Complex degradation



where R = receptor, P = penicillin, RP = inactive receptor-penicillin complex, RP* = inactive isomerized acyl-enzyme complex and X = chemically altered penicillin product.

^e Results from Fig. 3.

^f Results from Table 1.

^g clox, cloxacillin; amox, amoxicillin; het, hetacillin; ceph, cephalosporin; 6-apa, 6-aminopenicillanic acid.

^h PG binding to soluble or immobilized DDP in fortified bovine kidney or swine serum was examined when samples were either deproteinated with acetonitrile or not.

Table 3. Characteristics of fixed immobilized DDP

A	Residual activity of PG-DDP before immobilization	0%
B	Coupling efficiency	50%
C	Residual activity of immobilized PG-DDP	0%
D	Residual activity of immobilized PG-DDP + H ₂ O ₂	13%
E	Residual activity of immobilized DDP ('non-fixed' control)	2%

All residual activity values are relative to native, soluble DDP. DDP (150 nmol) was reacted with PG (1.04 μ mol) in 10 mM sodium phosphate buffer (pH 7) for 15 min at 37°C, after which unbound PG was removed by ultrafiltration. The retentate from the ultrafiltration step was assayed for residual enzyme activity with a tripeptide substrate (A), diluted to 1.5 mL with pH 4.6 buffer, immediately applied to 500 μ L Affi-Gel 10 in a micro-column and allowed to couple. Unbound PG-DDP was collected and assayed for protein concentration to determine coupling efficiency (B). The residual activity of the immobilized PG-DDP complex was measured (C). The gel was treated with 3% H₂O₂ as described in Table 1 and the enzyme activity again measured (D). The measured activity was compared with the activity of immobilized DDP which was not previously treated with PG (E).

13-fold. H₂O₂ was more effective in releasing the bound drug. This was in agreement with the BIAcore studies. Complete recovery of radioactivity was obtained with a 30 min incubation with H₂O₂ and the released drug was only diluted twofold.

A comparison of the ligand-binding behaviour of soluble and immobilized DDP is presented in Table 2.

Immobilization and PG-binding behavior of 'fixed' DDP

In an attempt to maintain the structural integrity of the active PG-binding site of DDP during immobilization, the enzyme was complexed with PG prior to the immobilization step. This immobilized enzyme is thus referred to as 'fixed'. Residual activity of the enzyme at various steps of the coupling procedure is shown in Table 3. Also shown are the coupling and PG binding efficiencies of the immobilized 'fixed' DDP. Complete activity loss is maintained when the PG-DDP complex is immobilized on the gel. Treatment of immobilized PG-DDP with H₂O₂ resulted in the recovery of 13 per cent of the DDP enzymatic activity compared with the free uninhibited enzyme. A 'non-fixed' immobilized DDP control (i.e. no incubation with PG prior to immobilization) showed little or no activity. HPLC analysis of the reaction sample between PG and 'fixed' immobilized DDP revealed extraneous peaks (retention times ca 5 and 9 min), possibly due to hydrolysis or degradation of the drug by DDP [Fig. 4(A)]. The native peak at ca 12 min represents ca 50 per cent of the DDP sites complexed. These peaks were not observed when PG was reacted with 'unfixed' immobilized DDP [Fig. 4(C)]. If PG was reacted with an aged gel matrix containing immobilized 'unfixed' DDP, these extra peaks (retention times ca 5 and 10 min) were again observed [Fig. 4(B)].

Discussion

The ligate binding and reactivity properties of *Streptomyces* DDP immobilized on a solid support by NHS chemistry is

summarized in Table 2. The enzyme has 14 Arg residues and 7 Lys residues (Duez *et al.*, 1987) which may be susceptible to reaction with NHS. Chemical modification and site-directed mutagenic experiments of *Streptomyces* DDP (Bourguignon-Bellefroid *et al.*, 1992; Hadonou *et al.*, 1992) demonstrated the significance of Arg⁹⁹ in the stabilization of enzyme structure and the role of Lys⁶⁵ in the tripeptide substrate and penicillin-binding capabilities of the enzyme. Other evidence support the hypothesis that an additional positively charged group (pK_a ≈ 9) in the enzymes active site may be involved in facilitating the binding of the penicillin molecule by forming an ion pair with the carboxylate of the antibiotic (Varetto *et al.*, 1987, 1991; Hadonou *et al.*, 1992). Clearly, reaction of any of these amino side-chains with NHS would have a detrimental effect on the enzymes reactivity with ligate. At first glance, it may appear that Lys⁶⁵ may be involved in the coupling linkage since catalytic activity with tripeptide substrate is virtually abolished when the enzyme is in the immobilized form (Eng *et al.*, 1995). However, since Hadonou *et al.*, 1992 observed the Lys⁶⁵ mutation to have an even greater drastic effect on penicillin binding than on catalytic activity, this amino acid residue is probably not a point of attachment to the support. Our other previous observation with iDDP was the differences in the pH profile for penicillin binding compared with sDDP. In these studies, we found that high pH (>8) did not affect the antibiotic binding ability of iDDP whereas an effect was seen for the soluble enzyme (Varetto *et al.*, 1987). A possible explanation for these observations may be that the basic protein group (pK_a ≈ 9.5) implicated in the studies by Varetto *et al.* may be reacted with NHS when the protein is immobilized and is therefore unavailable for binding to penicillin. This may account for the diminished ability for the solid-phase enzyme to bind antibiotic as well as for the immunity of the basic group toward changes in pH.

The kinetic studies of the inhibition of soluble DDP by penicillin by Nieto *et al.* (1973) and Frere *et al.* (1975) suggested that at low antibiotic concentrations [Scheme 1 (Table 2)], an equilibrium process describes the binding mechanism, where the dissociation constant K_d and reverse rate constant k_2 were determined to be 4.4 nM and $0.8 \times 10^{-4} \text{ s}^{-1}$, respectively. Based on the k_2 value and assuming dissociation of the complex follows first-order kinetics, its half-life is 2.4 h. At very high penicillin concentrations (>10-fold over the enzyme concentration), k_2 is no longer significant and the first part of the reaction becomes a rapid equilibrium process [Scheme 2 (Table 2)]. The following isomerization $\text{RP} \rightarrow \text{RP}^*$ occurs at enzymatic rates (179 s^{-1}) whereas the dissociation $\text{RP}^* \rightarrow \text{R} + \text{X}$ occurs at non-enzymatic rates ($0.21 \times 10^{-4} \text{ s}^{-1}$). The mechanisms are described in Table 2. Our observations (summarized in Table 2) indicates that the PG-iDDP complex dissociated reversibly from the enzyme after about a 2 h incubation at room temperature. Under the conditions of these experiments in which iDDP is incubated with an equimolar concentration of the antibiotic, our data are in agreement with Scheme 1. However, a dramatic decrease in affinity between the immobilized enzyme PG was also seen at the low antibiotic concentrations.

The binding kinetics of ampicillin with iDDP were also examined at high drug concentrations using surface plas-

mon resonance biosensor technology. A Scatchard analysis of the SPR response values at equilibrium at the various ampicillin concentrations revealed a K_d value of 4.95 mM. This is in good agreement with the K_d value (7.2 mM) obtained by Frere *et al.* (1975) using soluble DDP.

The reversal of the penicillin-iDDP interaction was examined using SPR and [14 C]penicillin G. These studies demonstrate the effectiveness of H_2O_2 for nucleophilic attack on the β -lactams carbonyl carbon involved in the acyl linkage with the enzyme. Similar observations were made for penicillin bound to particulate enzyme preparations from *Bacillus subtilis* (Lawrence and Strominger, 1970). Pen-

icillin-Sepharose has been used successfully in the purification of DD-peptidases and other penicillin-binding proteins from bacterial cell extracts, namely *B. subtilis* and *E. coli* B (Blumberg & Strominger, 1974; Gorecki *et al.*, 1975). Elution of the bound proteins from the penicillin affinity column was achieved in hydroxylamine and 0.5 M NaCl. However, we found hydrogen peroxide to be even better than hydroxylamine or salt in releasing bound drug.

Ever since their development in the early 1970s, immobilized enzymes have found widespread use in numerous preparative and analytical methods. The major advantages of using immobilized enzymes are that they can

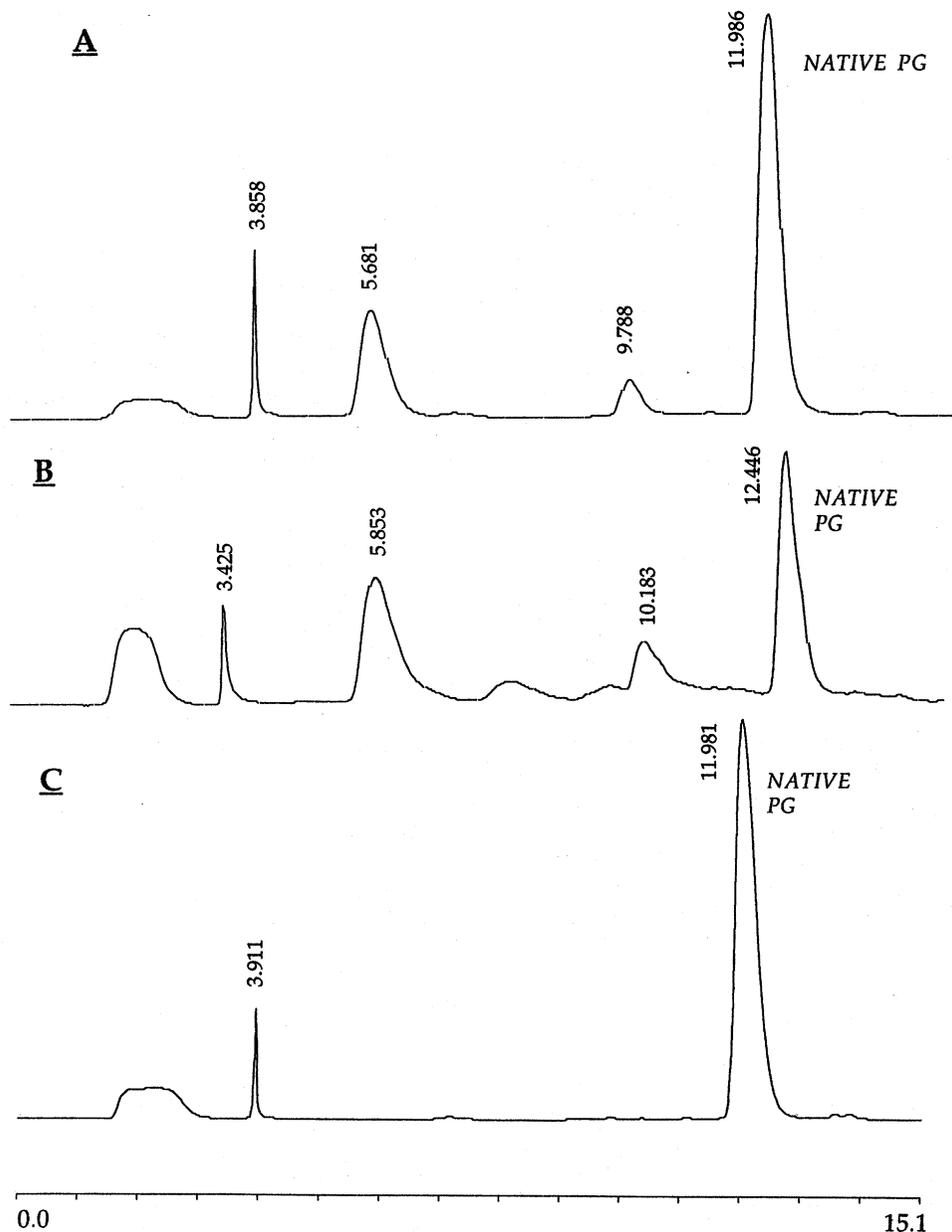


Figure 4. HPLC analysis of products formed from reaction of benzylpenicillin with immobilized 'fixed' DDP. PG (at an equal molar concentration to the immobilized enzyme) was incubated with 500 μ L of (A) immobilized 'fixed' DDP, (B) aged immobilized 'unfixed' DDP and (C) fresh immobilized 'unfixed' DDP for 15 min at 25°C in 10 mM sodium phosphate buffer (pH 7). The reaction solutions (500 μ L) were washed off the column, filtered through a 0.45 μ m membrane and applied to a PLRP-S HPLC column. UV detection was performed at 220 nm. The sensitivity was set at 0.001 a.u.f.s. and the flow rate was 1 mL/min.

be reused, resulting in considerable cost savings, and that the immobilized enzymes are often more stable than the free enzyme. Furthermore, the decay of enzyme stability usually becomes more predictable in the coupled form.

Covalent attachment and passive adsorption are the most commonly used methods for enzyme immobilization. It is desirable to retain full native enzyme reactivity with ligate as much as possible during coupling. The two types of protein groups (carboxyl and amino) most conveniently used for attachment to the support surface are also the most abundantly found in proteins. Thus, coupling chemistries targeting these groups would allow greater randomness of protein orientation on the surface, potentially resulting in blockage or inactivation of active sites. An even more undesirable consequence would be the involvement of protein groups participating in ligate binding in the coupling reaction. These drawbacks often lead to modifications in enzyme reactivity and diminished enzyme binding capacity and in many instances, even subtle alterations can have a substantial effect. This has been shown for numerous other enzymes (Lasch *et al.*, 1976; Sadana, 1992) and for DDP, described in this and earlier papers (Eng *et al.*, 1995). Changes in structural properties were also observed for immobilized monoclonal antibodies; however, unlike globular enzymes, these changes did not lead to loss of antigen binding (Kasche *et al.*, 1994). It would then be ideal to utilize an immobilization strategy which would minimize non-specific reactivity with various protein side-chains. Cys residues are generally present in low numbers in proteins and different immobilization chemistries and methodologies are available for specific reaction with the free thiol groups of cysteines (Hermanson *et al.*, 1993; Zull *et al.*, 1994). Unfortunately, the two Cys residues present in *Streptomyces* DDP exist as a disulfide-bonded pair (Joris *et al.*, 1987). Hence this approach for site-directed immobilization is not possible for DDP.

Because of the potential for structural changes to occur during immobilization, we attempted to preserve the active binding site of DDP during the coupling process. In this approach, the enzyme was first reacted with PG and then immobilized as a drug-bound complex. The goal was to lock or 'fix' the active site of DDP so that neither (1) groups important in the binding of analyte would be available for reaction with NHS nor (2) the tertiary structure in the

binding region would be deformed during the immobilization reaction. The increase in catalytic activity of immobilized 'fixed' DDP which we observe (Table 3) is possibly due to either an increase in its binding efficiency to substrate or to its enhanced flexibility for reaction on the bound substrate. Increased flexibility may also be responsible for the ability of the immobilized 'fixed' DDP to react on bound PG. The nature and quantitation of the breakdown products observed by HPLC remain to be determined.

It appears from the data that the binding of PG in the active site of DDP prior to immobilization may limit certain protein groups important for enzyme activity from covalent reaction with the gel matrix. Likewise, in old gel preparations containing iDDP, certain contacts between the enzyme and gel may be lost with time allowing the enzyme to experience increased flexibility. In either case, the immobilized enzyme has an enhanced ability to react on the bound PG by either degrading or hydrolyzing it. Another critical factor for ligand binding is the random orientation of the enzyme when it is immobilized to the solid support resulting in blocked binding sites. Methods that can predictably couple the enzyme so that its binding site is still fully accessible to potential ligands need to be investigated.

We have taken a fundamental step in our attempt to investigate bioaffinity techniques for penicillin residues and that is to understand better the biomolecular interactions governing the affinity between two species, one of which is chemically linked to a support matrix. The combined data from our earlier work and the present account show that immobilization of *Streptomyces* R61 DDP by reaction with the enzymes susceptible amino groups resulted in changes in catalytic activity on a tripeptide substrate, penicillin-binding efficiencies, penicillin affinity at low drug concentrations and pH stability of drug binding. Factors studied which were unaffected by immobilization were the penicillin affinity at high drug concentrations, drug-enzyme complex stability, binding reaction mechanism, drug selectivity and method of complex desorption.

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