Photoaffinity labelling of isopenicillin N synthetase by laser-flash photolysis

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Isopenicillin N synthetase (IPNS) from *Acremonium chrysogenum* was photolabelled by laser-flash photolysis in the presence of a diazirinyl-containing substrate, 2-[3-(3-trifluoromethyl-3H-diazirin-3-yl)-phenoxy]acetyl-S-methyloxycarbonylsulphenyl-L-cysteinyl-D-valine (DCV). Labelling of IPNS by DCV is partially inhibited in the presence of an excess of L- α -amino adipoyl-L-cysteinyl-D-valine (ACV), the natural substrate. In the absence of light, DCV is converted into the corresponding penicillin with comparable K_m but significantly depressed V_{max} relative to ACV. Selective incorporation of [14 C]DCV into IPNS has been demonstrated by fluorography of IPNS analysed by SDS/polyacrylamide-gel electrophoresis. Scintillation counting of labelled IPNS purified on an ion-exchange f.p.l.c. column confirms this result. This methodology may be applicable for studies aimed at investigating the binding of substrates to IPNS.

INTRODUCTION

Isopenicillin N synthetase (IPNS) is the enzyme responsible for the conversion of L- α -amino adipoyl-L-cysteinyl-D-valine (ACV) (Ia) into isopenicillin N (IIa) by both filamentous fungi (e.g. *Acremonium chrysogenum*) and streptomycetes (Scheme 1) [1]. In studies aimed at understanding the nature of the enzyme-catalysed reaction, IPNS has been shown to convert a wide variety of analogue substrates into β -lactams, thereby providing routes to a range of new antibiotics [1]. IPNS has been cloned and expressed in an active form at high concentrations in *Escherichia coli* (approx. 20% of total protein) [2]. Although its amino acid sequence is known, there are no data currently available about the three-dimensional structure of IPNS.

It has previously been shown that analogues of ACV with aromatic side chains [e.g. phenoxyacetyl-L-cysteinyl-D-valine (VCV) (Ib)] are substrates for IPNS and that these substrates have similar Michaelis constants (K_m) to

the native substrate ACV but considerably smaller maximal rate parameters (V_{max}); under identical incubation conditions this results in poor conversion yields [3]. However, aryl-substituted substrates are more readily accessible, and produce β -lactams of greater commercial significance than amino adipate-derived peptides. We are therefore interested in altering the substrate specificity of IPNS via mutagenesis to favour aryl-substituted peptide substrates, and are investigating photoaffinity labelling as a means of providing relevant structural information for this and other aspects of IPNS research.

Photoaffinity labelling [4] was chosen as a suitable technique for the identification of the side-chain-binding site of IPNS. Photolysis of photoaffinity reagents generates a highly reactive species that labels regions of the protein in contact with the bound inhibitor, irrespective of the nature of the amino acid residues present. Although azides have been widely used for the photoaffinity labelling of enzymes [4], they are readily reduced by thiols, so that their application to labelling studies of IPNS, which requires a reducing environment to remain active, was inappropriate. Diazirines, however, have found widespread utility in such work, since they are readily accessible, are chemically and thermally inert, are stable to physiological conditions and can be cleanly photolysed at wavelengths in the near u.v. [4]. Their suitability for studies with IPNS was evident, and 2-[3-(3-trifluoromethyl-3H-diazirin-3-yl)phenoxy]acetyl-S-methyloxycarbonylsulphenyl-L-cysteinyl-D-valine (DCV) (III) has been synthesized as a potential photoaffinity inhibitor of IPNS [5]. We here report the kinetic data for conversion of DCV into the corresponding penicillin (IV) and the successful photoaffinity labelling of IPNS with this reagent.

Scheme 1. Conversion of dipeptide substrates (I) into penicillins (II) by IPNS

Abbreviations used: ACV, L- α -amino adipoyl-L-cysteinyl-D-valine; VCV, phenoxyacetyl-L-cysteinyl-D-valine; IPNS, isopenicillin N synthetase; DCV, 2-[3-(3-trifluoromethyl-3H-diazirin-3-yl)phenoxy]acetyl-S-methyloxycarbonylsulphenyl-L-cysteinyl-D-valine; [14 C]DCV, 2-[3-(3-trifluoromethyl-3H-diazirin-3-yl)phenoxy][14 C]acetyl-S-methyloxycarbonylsulphenyl-L-cysteinyl-D-valine; DTT, dithiothreitol.

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MATERIALS AND METHODS

Materials

DCV was prepared according to the literature method [5], and reduced to the free thiol *in situ* with dithiothreitol (DTT). DTT and Trizma base were from Sigma Chemical Co., Poole, Dorset, U.K. ACV and 6-aminopenicillanic acid were gifts from Eli Lilly and Co., Indianapolis, IN, U.S.A., and other chemicals were from BDH Chemicals, Poole, Dorset, U.K., or Aldrich Chemical Co., Gillingham, Dorset, U.K.

Irradiations

Irradiations were performed with a mercury source (Mineralight lamp) at 254 nm, or a frequency-tripled neodymium-YAG laser at 355 nm (System 2000; SK Lasers, Cambridge, U.K.). Quartz cells, either 1 mm or 1 cm path-length, were used for the irradiations. The power output of the laser at 355 nm was measured with a Photon Control (Cambridge, U.K.) power meter, and found to be 21 ± 2 mJ/pulse, at a repetition rate of 10 Hz.

Measurement of radioactivity

Radioactivity measurements were made by liquid-scintillation counting with an LKB 1215 Rackbeta instrument and LKB Optisafe scintillation fluid.

Preparation of cell-free extracts containing IPNS

IPNS, isolated from *Acremonium chrysogenum*, was prepared according to the published procedure [6]. This fungal enzyme had a specific activity of 0.023 unit ($\mu\text{mol}/\text{min}$)/mg, and was estimated to be approx. 60% pure by SDS/polyacrylamide-gel electrophoresis. Recombinant IPNS, cloned from *A. chrysogenum*, was isolated from *E. coli* by the published procedure [7]. This partially purified IPNS was obtained with a specific activity of 0.45 unit/mg, and was estimated to be approx. 80% pure by SDS/polyacrylamide-gel electrophoresis. The molecular mass of IPNS is 38 kDa.

Substrate incubation with IPNS

The reaction mixture, containing enzyme, the substrate (ACV or DCV, 0.38 mM), DTT (11 mM), ascorbic acid (1.1 mM) and FeSO_4 (0.1 mM) in 50 mM-ammonium bicarbonate buffer, pH 7.9, with a final volume of 300 μl , was incubated at 27 °C in an orbital incubator, shaking at 250 rev./min, for 10–30 min. The reaction was quenched by the addition of acetone (300 μl), which precipitated the protein. The sample was centrifuged, and the acetone was removed from the supernatant by a stream of air. A 100 μl portion from the supernatant was then bioassayed, as described below.

Bioassay of penicillin products

Microbiological assays were performed by the hole-plate method [8] with the test organism *Staphylococcus aureus* N.C.T.C. 6571. Solutions (100 μl) to be tested for penicillin content were loaded into wells in bioassay plates, and incubated overnight at 37 °C. The diameters of the resultant inhibition zones were measured, and amounts of product were estimated by reference to standards prepared with cephalosporin C.

SDS/polyacrylamide-gel electrophoresis

Analytical electrophoresis was carried out by the

method of Laemmli [9] in a slab-gel apparatus (Bio-Rad Laboratories, Richmond, CA, U.S.A.) with 10% (w/v) polyacrylamide gels containing SDS (0.1%). Gels were stained with Coomassie Brilliant Blue R-250 dye.

Fluorography

After being destained, the SDS/polyacrylamide gels were soaked in Amplify (Amersham) and dried. The gel was then fluorographed on Fuji X-Ray film at -78 °C for 14 weeks.

Synthetic methods

Standard chemical procedures as previously described were used [5]. Melting points were recorded on a Buchi 510 apparatus and are uncorrected. I.r. spectra were recorded on a Perkin-Elmer model 681 spectrophotometer. U.v. spectra were recorded either on a Pye-Unicam PU 8800 UV-VIS spectrophotometer or a Perkin-Elmer 555 UV-VIS spectrophotometer. ^1H -n.m.r. spectra were recorded on a Bruker WH 300 MHz or AM 250 MHz spectrometer. Mass spectra were recorded on VG Analytical ZAB 1F MM 30F mass spectrometers.

Synthesis of benzhydryl iodoacetate (VI)

A solution of iodoacetic acid (500 mg, 2.8 mmol) in acetone (10 ml) was treated with diphenyldiazomethane (650 mg, 3.4 mmol) [10] in acetone (10 ml), and the mixture was stirred at room temperature for 2 h. The solvent was removed and the crude material was purified by flash chromatography [11] [dichloromethane/light petroleum (b.p. 60–80 °C) (1:10, v/v, then 1:1 v/v)] to give the *title compound* (VI) as a colourless oil (780 mg, 80% yield). $\text{C}_{15}\text{H}_{13}\text{IO}_2$ requires C, 51.2; H, 3.72% (Found C, 51.1; H, 3.76%). ^1H n.m.r.: δ (p.p.m.) (300 MHz, $[\text{^2H}]\text{chloroform}$) 3.69 (2H, s, ICH_2), 6.78 (1H, s, CHPh_2) and 7.16–7.30 (10H, m, Ar H). ^{13}C n.m.r.: δ (p.p.m.) (62.85 MHz, $[\text{^2H}]\text{chloroform}$) –5.4 (t, CH_2I), 78.5 (d, CHPh_2), 127.1, 128.1, 128.5 (all d, Ar C), 139.4 (s, Ar C) and 167.5 [s, $\text{C}(\text{O})\text{O}$]. I.r. (chloroform): 1730 (s), 1495 (m), 1450 (m), 1415 (m), 1260 (s), 1085 (s), 980 (m) and 700 (s) cm^{-1} . M.s.: m/z (field desorption) 352 (M^+).

Synthesis of benzhydryl 3-(3-trifluoromethyl-3H-diazirine-3-yl)phenoxyacetate (VII)

Benzhydryl iodoacetate (VI) (293 mg, 0.83 mmol), 3-(3-hydroxyphenyl)-3-trifluoromethyl-3H-diazirine [5] (140 mg, 0.69 mmol) and K_2CO_3 (143 mg, 1.0 mmol) in acetone (5 ml) were stirred overnight at room temperature. The solvent was removed, and the crude product was purified by flash chromatography [dichloromethane, then dichloromethane/light petroleum (b.p. 60–80 °C) (1:1, v/v)] to give the *title compound* (VII) as a colourless oil (360 mg, 99% yield). $\text{C}_{23}\text{H}_{17}\text{F}_3\text{N}_2\text{O}_3$ requires C, 64.8; H, 4.0; N, 6.6% (Found C, 65.0; H, 4.0; N, 6.7%). ^1H n.m.r.: δ (p.p.m.) (300 MHz, $[\text{^2H}]\text{chloroform}$) 4.75 (2H, s, OCH_2), 6.75 (1H, s, $H-2$), 6.86 (1H, ddd, $J = 8.4$ Hz, $H-4$ or $H-6$), 6.92 (1H, ddd, $J = 7.5$ Hz, $H-6$ or $H-4$), 7.04 (1H, s, CHPh_2) and 7.27–7.40 (11H, m, Ar H). ^{13}C n.m.r.: δ (p.p.m.) (62.85 MHz, $[\text{^2H}]\text{chloroform}$) 65.5 (t, OCH_2), 78.0 (d, OCHPh_2), 113.3 (d), 115.7 (d), 119.8 (d), 124.2 (s), 127.1 (d), 128.2 (d), 128.6 (d), 130.1 (d), 130.8 (s), 139.3 (s), 158.0 (s) and 167.5 [s, $\text{C}(\text{O})\text{O}$]. I.r. (chloroform): 1760 (s), 1605 (m), 1580 (m), 1495 (m), 1450 (m), 1435 (w), 1350 (w), 1340 (w), 1265 (m), 1245

(m), 1085 (m) and 700 (s) cm^{-1} . U.v. λ_{max} (methanol): 268 nm ($\log \epsilon$ 3.2) and 252 nm ($\log \epsilon$ 2.4). M.s.: m/z (field desorption) 426 (M^+).

Synthesis of 3-(3-trifluoromethyl-3H-diazirin-3-yl)-phenoxyacetic acid (VIII)

To the ester (VII) (100 mg, 0.23 mmol) was added anisole (10 mg, 0.09 mmol), redistilled trifluoroacetic acid (2 ml) and dichloromethane (2 ml), and the mixture was stirred at room temperature for 20 min. The solvent was removed *in vacuo*, and the residue was treated twice with toluene, and dried under vacuum. The crude product was purified by flash chromatography [dichloromethane, then methanol/dichloromethane (1:10, v/v)] to give the *title compound* (VIII) as a white solid (30 mg, 50% yield), m.p. 75–80 °C. ^1H n.m.r.: δ (p.p.m.) (300 MHz, $[\text{H}]$ -chloroform) 4.50 (2H, s, OCH_2), 6.66 (1H, s, $H-2$), 6.80 (1H, d, $J = 7.8$ Hz, $H-4$ or $H-6$), 6.85 (1H, d, $J = 8.2$ Hz, $H-6$ or $H-4$) and 7.23 (1H, dd, $J = 8.0$ Hz, $J' = 8.0$ Hz, $H-5$). ^{13}C n.m.r.: δ (p.p.m.) (62.85 MHz, $[\text{H}]$ -chloroform/ $[\text{H}]$ -methanol) 65.4 (t, OCH_2), 113.5 (d), 119.7 (s and d), 124.2 (s), 130.1 (d), 130.7 (s), 158.0 (s) and 171.8 [s, C(O)OH]. I.r. (chloroform): 3000 (b), 1740 (s), 1610 (s), 1585 (s), 1495 (m), 1435 (m), 1345 (m), 1295 (m), 1250 (m) and 1080 (m) cm^{-1} . U.v. λ_{max} (methanol): 274 nm ($\log \epsilon$ 3.2) and 352 ($\log \epsilon$ 2.44). M.s.: m/z (fast atom bombardment) 260 (M^+ , 10%), 232 (54%), 175 (46%), 174 (28%), 157 (100%), 145 (53%), 138 (45%), 131 (54%), 127 (32%), 115 (65%), 109 (22%), 107 (37%), 103 (37%), 87 (24%), 75 (28%), 69 (30%), 63 (27%) and 57 (47%).

Synthesis of (2*S*,5*R*,6*R*)-1-aza-3,3'-dimethyl-7-oxo-6-[3-(3-trifluoromethyl-3H-diazirin-3-yl)phenoxyacetamido]-4-thiabicyclo[3.2.0]heptane-2-carboxylic acid (IV)

Acid (VIII) (30 mg, 0.12 mmol) in dry toluene (3 ml) and dimethylformamide (2 drops) was treated with oxalyl chloride (18 mg, 0.14 mmol) at 0 °C, and stirred for 1 h. The solvent was removed *in vacuo*, and the residue was dissolved in acetone (3 ml).

To a solution of 6-aminopenicillanic acid (25 mg, 0.12 mmol) in 4% (w/v) KHCO_3 (3 ml) and acetone (3 ml) was added dropwise with stirring and cooling the above acid chloride solution over 10 min, and the solution was stirred at 0 °C for 1 h. The solution was freeze-dried, redissolved in 4% KHCO_3 solution, and extracted with dichloromethane. The aqueous phase was layered with dichloromethane, then acidified to pH 2 (5 M- H_2SO_4), and the layers were separated. The aqueous phase was washed with dichloromethane, dried over anhydrous Na_2SO_4 and the solvent was removed, to give the *title compound* (IV) (40 mg, 75% yield.). ^1H n.m.r.: δ (p.p.m.) (300 MHz, $[\text{H}]$ -chloroform) 1.61 (6H, s, 2CH_3), 1.65 (3H, s, CH_3), 4.50 (1H, s, $H-3$), 4.58 (1H, d, OCH_2), 4.60 (1H, d, OCH_2), 5.58 (1H, d, $J = 4.3$ Hz, $H-5$), 5.74 (1H, dd, $J = 4.3$ Hz, $J = 9.1$ Hz, $H-6$), 6.76 (1H, s, $H-2'$), 6.88 (1H, d, $J = 7.8$ Hz, $H-4'$ or $H-6'$), 6.98 (1H, dd, $J = 2.5$ Hz, $J' = 6.9$ Hz, $H-6'$ or $H-4'$), 7.37 (1H, dd, $J = 8.0$ Hz, $J' = 7.2$ Hz, $H-5'$) and 9.01 (2H, bs, CO_2H and NH). I.r. (chloroform): 3400 (m), 3020 (m), 1785 (s), 1740 (s), 1690 (s), 1610 (s), 1585 (s), 1520 (s), 1440 (m), 1290 (s), 1080 (m) and 690 (m) cm^{-1} . U.v. λ_{max} (methanol): 274 nm ($\log \epsilon$ 3.17) and 352 nm ($\log \epsilon$ 2.30). M.s.: m/z (fast atom bombardment) 459 ($M^+ + 1$, 2%), 175 (15%), 160 (100%), 157 (77%), 137 (24%), 114 (38%), 87 (23%), 70 (26%), 69 (23%) and 55 (21%).

Synthesis of iodo[2- ^{14}C]acetyl-(*S*-diphenylmethyl)-L-cysteiny-D-valine benzhydryl ester

To a solution of *S*-(diphenylmethyl)-L-cysteiny-D-valine benzhydryl ester [5] (70 mg, 0.13 mmol), iodoacetic acid (20 mg, 0.11 mmol) and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (40 mg, 0.16 mmol) in dichloromethane (1 ml) was added iodo[2- ^{14}C]acetic acid (50 μCi) in dichloromethane (2 ml), and the mixture was stirred at room temperature overnight. The reaction mixture was diluted with dichloromethane, washed with saturated aqueous NaHCO_3 solution and with 10% (w/v) citric acid solution, and dried over anhydrous Na_2SO_4 . The solvent was removed *in vacuo*, and the crude product was purified by flash chromatography [dichloromethane, then ethyl acetate/dichloromethane (1:20, v/v)] to give the *title compound* as a white solid (73 mg, 96% yield), which was identical by t.l.c. with an authentic sample [5] [R_F 0.5 in ethyl acetate/dichloromethane (1:20, v/v)].

Synthesis of 2-[3-(3-trifluoromethyl-3H-diazirin-3-yl)-phenoxy][2- ^{14}C]acetyl-*S*-carbomethoxysulphenyl-L-cysteiny-D-valine (^{14}C DCV)

The above dipeptide (73 mg) was converted into the *title compound* [^{14}C]DCV in three steps according to the literature method [5] in 80% overall yield. The radiochemical specific activity of the product [^{14}C]DCV was found to be 240 mCi/mol, giving a radiochemical yield of 40% from iodo[2- ^{14}C]acetic acid. A u.v. spectrum of [^{14}C]DCV showed the characteristic diazine absorption at 350 nm.

Incubation of [^{14}C]DCV with IPNS and cofactors under the normal conditions followed by bioassay produced an inhibition zone on *S. aureus* plates, with the corresponding penicillin produced in 0.04% yield.

Determination of K_m and V_{max} data for the conversion of DCV by IPNS

K_m and V_{max} data were obtained by the method of Baldwin *et al.* [3]: A mixture of IPNS (0.75 unit), DCV (0.33–1.6 mM), DTT (18 mM), ascorbic acid (1.8 mM) and FeSO_4 (0.18 mM) in 50 mM-ammonium bicarbonate buffer, pH 7.9 (total volume 550 μl), was incubated at 27 °C and shaken at 260 rev./min for 5 min. Samples (100 μl) were withdrawn after 40 s, 60 s, 90 s, 120 s and 300 s intervals, and immediately added to acetone (100 μl) to quench the reaction. The precipitated protein was separated by centrifugation, and the acetone was removed by a stream of air. The supernatant was adjusted to 100 μl total volume with 50 mM-ammonium bicarbonate buffer, pH 7.9, and then bioassayed.

Photolysis of DCV

The concentration of DCV in solutions during photolyses was determined from the intensity of the diazine absorption at 350 nm in the u.v. spectrum. This absorption decreases on decomposition of the diazine group (see Fig. 1).

(i) A solution of DCV (2.5 mM) and DTT (8.4 mM) in 28 mM-Tris/HCl buffer, pH 7.7, and 14 mM-ammonium bicarbonate buffer, pH 7.9 (total volume 357 μl), was irradiated at 254 nm by the Mineralight lamp, over a 2 h period at 4 °C. See Fig. 1.

(ii) A solution of DCV (6 mM) and DTT (8.4 mM) in 32 mM-ammonium bicarbonate (total volume 157 μl), was irradiated at 355 nm by the neodymium-YAG laser

at 10 °C over 10 min. During irradiation, N₂ gas was seen to be evolved. See Fig. 1.

Photo-inactivation of IPNS by DCV

A pair of incubation mixtures was prepared. Both contained DTT (22 mM) and recombinant IPNS (0.07 mg) in 50 mM-ammonium bicarbonate buffer, pH 7.9 (total volume 157 µl); one contained DCV (6 mM) and the other did not. After preincubation at 0 °C for 10 min, both of these mixtures were irradiated at 355 nm (neodymium-YAG laser) for 6 min. Another pair of samples were also prepared as above, except that, instead of being irradiated, they were kept in the dark. The enzyme was subsequently concentrated by centrifugation in Amicon Centricon-10 microconcentrators (5000 *g* for 1 h at 4 °C), and washed twice with 50 mM-ammonium bicarbonate (50 µl). The enzyme solution was then diluted to 200 µl with 50 mM-ammonium bicarbonate; 180 µl of this solution was incubated with a mixture containing ACV disulphide (1.6 mM), DTT (10.5 mM), ascorbic acid (1.1 mM) and FeSO₄ (0.11 mM) and made up to a total volume of 330 µl with 50 mM-Tris/HCl buffer, pH 7.7. The solution was shaken at 27 °C for 15 min, and the yield of isopenicillin N was determined by bioassay in the usual manner. These values were used to calculate the extent of loss of activity of IPNS during irradiation in the absence or in the presence of DCV.

The dependence of photoinactivation on DCV concentration was determined by comparing the IPNS activity (by bioassay) of both irradiated and non-irradiated samples at different concentrations of DCV. Samples of IPNS were photoinactivated as described above, except that the DCV concentration was varied between 0 and 5.7 mM.

Protection of IPNS by ACV against photo-inactivation by DCV

Mixtures containing DTT (63 mM), recombinant IPNS (140 µg) and DCV (5.6 mM), either with or without ACV (74 mM), were irradiated. The IPNS was preincubated with the ACV and DTT before the addition of DCV. A 250-fold excess of DCV over IPNS was used. An additional pair of control samples were prepared, but kept in the dark. Each enzyme solution was then concentrated, washed and assayed as described above.

Incorporation of [¹⁴C]DCV into IPNS

In a typical experiment, a mixture containing [¹⁴C]DCV (5.6 mM), DTT (45 mM) and fungal IPNS (0.20 mg) in 50 mM-ammonium bicarbonate buffer, pH 7.9 (total volume 330 µl), was prepared. Two fractions were taken (150 µl each), one of which was irradiated for 7.5 min (neodymium-YAG laser) and the other of which was kept in the dark. Each of these solutions contained 0.1 µCi of ¹⁴C radioactivity. The molar ratio of [¹⁴C]DCV to IPNS was 360:1. The protein in both solutions was then concentrated by centrifugation in Amicon Centricon-10 microconcentrators (5000 *g* for 1 h at 4 °C), and washed seven times with DTT (250 mM) in 50 mM-ammonium bicarbonate. The ¹⁴C content of the washings was determined by the addition of Optisafe (3 ml) followed by liquid-scintillation counting. The radioactive content of the last wash filtrate was less than 0.5% of the ¹⁴C initially added. The protein was isolated, and ex-

amined by SDS/polyacrylamide-gel electrophoresis and fluorography. See Fig. 2.

Photoaffinity labelling of IPNS by [¹⁴C]DCV

In a typical experiment, a mixture containing [¹⁴C]DCV (0.71 mM), DTT (91 mM) and recombinant IPNS (2.0 mg) in 50 mM-ammonium bicarbonate buffer, pH 7.9 (total volume 330 µl), was prepared. Two fractions were taken (150 µl each), one of which was irradiated for 7.5 min (neodymium-YAG laser) and the other of which was kept in the dark. Each of these solutions contained 0.025 µCi of ¹⁴C radioactivity (specific radioactivity 240 mCi/mol). The molar ratio of DCV to IPNS was 4.7:1. After this treatment the protein in both solutions was concentrated by centrifugation in Amicon Centricon-10 microconcentrators (5000 *g* for 1 h at 4 °C), and washed seven times with DTT (250 mM) in 50 mM-ammonium bicarbonate. The ¹⁴C content of the washings was determined by liquid-scintillation counting. The radioactivity content of the last wash filtrate was less than 1.2% of the ¹⁴C initially added. The protein was then purified by ion-exchange chromatography on a Pharmacia f.p.l.c. system. A Mono Q 5/5 anion-exchange column was used, developed at a flow rate of 1 ml/min at room temperature, with a linear gradient of 0–220 mM-NaCl in 20 mM-Tris/HCl buffer, pH 7.7, and the eluate was monitored at 280 nm. The radioactivity content of the eluate fractions (1 ml) was determined by the addition of Optisafe (6 ml) followed by liquid-scintillation counting. The results are shown in Fig. 3. The total radioactivity content of this modified IPNS, contained in fractions 22–25, was 11.7 pCi for the non-irradiated control, and 132 pCi for the irradiated sample. From the *A*₂₈₀ data, the protein recovery was calculated to be 109 µg (assuming that *A*₂₈₀^{1%} = 8.6). This corresponds to a specific radioactivity of 46 mCi/mol for the labelled IPNS. This represents a 19% incorporation of DCV into IPNS, calculated on a mole-for-mole basis.

Protection of IPNS by ACV against photoaffinity labelling by [¹⁴C]DCV

A mixture containing ACV (0, 1.45 or 14.5 mM), DTT (152 mM) and recombinant IPNS (2.0 mg) in 50 mM-ammonium bicarbonate buffer, pH 7.9 (total volume 280 µl), was preincubated at 20 °C for 5 min. [¹⁴C]DCV (final concentration 0.71 mM) was added to give a total volume of 330 µl, and each of the solutions divided into two fractions (150 µl each), one of which was irradiated for 7.5 min (neodymium-YAG laser) and the other of which was kept in the dark. Each of these solutions contained 0.025 µCi of ¹⁴C radioactivity (specific radioactivity 240 mCi/mol). The molar ratio of DCV to IPNS was 4.7:1. The protein was purified by washing in Amicon Centricon-10 microconcentrators, followed by separation by ion-exchange f.p.l.c., as described above. The eluate was monitored at 280 nm. The radioactivity content of the eluate fractions (1 ml) was determined by the addition of Optisafe (6 ml) followed by liquid-scintillation counting. The amount of IPNS protein eluted from the ion-exchange column was determined from the area of the *A*₂₈₀ peak corresponding to IPNS. The incorporation of radioactivity per unit of IPNS protein was calculated for each sample at 0 mM, 1.45 mM or 14.5 mM ACV concentrations with or without irradiation.

Scheme 2. Conversion of DCV (III) into penicillin (IV) by IPNS

RESULTS AND DISCUSSION

DCV was shown to be a substrate for IPNS in the following manner. Incubation of DCV and IPNS in the absence of u.v. irradiation gave rise to a product that was antibiotically active against *S. aureus* by a hole-plate assay, and sensitive to penicillinase (*Bacillus cereus* I). DCV incubated in the presence or in the absence of cofactors and boiled IPNS showed no bioactivity. These results are consistent with enzyme-mediated formation of the expected penicillin (IV) (Scheme 2).

An authentic sample of penicillin (IV) was prepared by acylation of 6-aminopenicillanic acid with the appropriate acyl chloride (Scheme 3). The biological activity of authentic penicillin (IV) was equivalent to that of the incubation product and allowed quantification of the conversion yield of DCV. Antibiotic activity against *S. aureus* was found to be one-tenth that of penicillin V and 100 times that of cephalosporin C. The enzymic conversion yield of DCV was 0.25% relative to the conversion of ACV; the kinetic parameters of the conversion were determined by a stopped bioassay technique [3]. These results, which are shown in Table 1, indicate that substitution of the aromatic ring by the photolabile group does not significantly affect the yield from the enzymic conversion (entries 2 and 3). However, both phenoxyacetyl-L-cysteinyl-D-valine and DCV gave much lower conversion yields than the natural substrate ACV (entry 1, Table 1).

U.v. irradiation of DCV with a mercury lamp at

Table 1. Yields and kinetic data for enzymic conversion of various substrates by IPNS into the corresponding penicillins

Entry	Substrate	Yield (%)	K_m (mM)	V_{max} (mmol/min per μ mol)
1	ACV	100	0.16	1.64
2	VCV	0.04	0.50	0.80×10^{-3}
3	DCV	0.25	0.77	0.75×10^{-3}

254 nm showed approximate first-order decomposition with a half-life of approx. 30 min (Fig. 1). However, this method of photolysis proved unsatisfactory in subsequent experiments, since the prolonged exposure to this wavelength (required to produce complete decomposition of DCV) caused up to 80% loss of the catalytic activity of IPNS. Although literature reports have described extensive studies of the decomposition of diazirines to the corresponding carbenes by using laser-flash photolysis [12,13], this technique has not been widely used for photoaffinity labelling studies [14–18]. A frequency-tripled neodymium-YAG laser provides high-intensity monochromatic light at 355 nm, corresponding closely to the diazirine absorption maximum (λ_{max} , 350 nm) but not to that of proteins (λ_{max} , approx. 280 nm), and therefore offers the possibility of selective generation of the reactive carbenes. Laser-flash photolysis (pulse energy 21 ± 2 mJ, repetition rate 10 Hz) of DCV gave complete decomposition of the diazirine group in 7 min under conditions that had no effect on IPNS activity (Fig. 1). Under these conditions IPNS was inactivated upon irradiation at 355 nm in the presence of DCV and dithiothreitol (DTT), and at least a 250-fold

Scheme 3. Synthesis of an authentic sample of penicillin (IV)

Abbreviation: 6-APA, 6-aminopenicillanic acid.

Fig. 1. Photolyses of DCV solutions

□, Mercury-lamp photolysis at 254 nm; ♦, frequency-tripled neodymium-YAG laser at 355 nm. For details see the text.

molar excess of DCV over IPNS was required in order to achieve complete inactivation.

The relatively low affinity of DCV for IPNS, as indicated by its K_m value (entry 3, Table 1), necessitates the use of a large excess of the labelling reagent in order to achieve complete inactivation of the protein. The possibility of non-specific labelling of the enzyme [4] under these conditions was evaluated by conducting additional photolysis experiments in the presence of the natural substrate ACV. In such an experiment the label, DCV, is expected to be displaced competitively, leading to a decrease in active-site specific labelling [4]. When a mixture containing ACV, DCV, DTT and IPNS was incubated under the normal irradiation conditions (the ratio of ACV to DCV was 13:1, and the ratio of DCV to IPNS was 4.7:1), bioassay showed that 48% of the activity of IPNS was retained, compared with its activity in a non-irradiated sample. Under the same conditions, but in the absence of ACV, complete inactivation of IPNS resulted. The most likely explanation for these results is that ACV is protecting the active site from labelling by DCV. However, this protection by ACV is not complete. It is possible that there is residual non-specific labelling of the protein by DCV at a site distant from the active site but that nonetheless causes loss of enzyme activity [4].

[^{14}C]DCV was then used to demonstrate photoaffinity labelling of IPNS directly. Irradiation of IPNS in the presence of [^{14}C]DCV and 100 mM-DTT was followed by concentration and desalting of the mixture in an Amicon Centricon-10 microconcentrator (10000 Da molecular-mass cut-off). Thorough washing with 250 mM-DTT was carried out to remove non-covalently bound [^{14}C]DCV. A non-irradiated control was also prepared and the IPNS protein was washed as described above. Fluorography of SDS/polyacrylamide gels of samples from both the irradiated and the non-irradiated mixtures indicated that IPNS from the irradiated sample showed incorporation of radioactivity, i.e. photolabelling of IPNS had occurred (Fig. 2). This labelling of IPNS was selective, although it was not totally specific, since only IPNS and one other protein in the partially purified fungal extract (which contained six other protein species)

Fig. 2. Incorporation of [^{14}C]DCV into IPNS

Fungal IPNS was incubated with [^{14}C]DCV and DTT, and either kept in the dark (–) or irradiated (+) with a neodymium-YAG laser. Washed protein samples were freeze-dried, separated by SDS/polyacrylamide-gel electrophoresis, stained with Coomassie Brilliant Blue (a) and then fluorographed at -78°C for 14 weeks (b). For details see the text. M, molecular-mass markers: bovine serum albumin (66.2 kDa), hen's-egg ovalbumin (45 kDa), bovine erythrocyte carbonic anhydrase (30 kDa), horse myoglobin (17.2 kDa) and horse cytochrome *c* (12.3 kDa).

were labelled under these conditions. The heavy band appearing in the fluorogram at the gel front is not protein, since it does not stain with Coomassie Brilliant Blue dye. Similar examples of the incomplete specificity of photoaffinity labels for the targets for which they were designed have been reported [19–21].

In order to confirm that the incorporation of radioactivity into IPNS occurs only in the irradiated sample under these conditions, an alternative approach was adopted. In this experiment, mixtures containing IPNS and [^{14}C]DCV (both irradiated and non-irradiated, as described above, but with the ratio of DCV to IPNS being 4.7:1) were thoroughly washed in Amicon Centricon-10 microconcentrators, and the enzyme was isolated and purified by ion-exchange f.p.l.c. A radioactively labelled protein was isolated, which was eluted in the position expected for IPNS, and which appeared identical to both untreated IPNS and to IPNS from the non-irradiated control sample when examined by SDS/polyacrylamide-gel electrophoresis. The data are shown in Fig. 3; in the non-irradiated solution (– $h\nu$) there has been no uptake of radioactivity into the IPNS. The irradiated sample (+ $h\nu$) clearly demonstrates radiochemical incorporation into IPNS. Complete inhibition of IPNS activity required photolysis in the presence of a 250-fold excess of DCV. Radiochemical incorporation experiments were carried out with a lesser excess (4.7:1) of DCV to minimize non-specific labelling. Under these conditions the specific radioactivity of the labelled IPNS was found to be 46 mCi/mol, corresponding to a 19% incorporation of the label into the protein on a molar basis. This may be due to the presence of inactive IPNS in the enzyme pool.

As in the experiments with non-radioactive DCV, the specificity of labelling by [^{14}C]DCV was examined by performing photolyses in the presence of various concentrations of the natural substrate ACV. The degree of radiochemical incorporation was decreased by 24% in the presence of a 2-fold excess of ACV (relative to [^{14}C]DCV) and by 59% with a 20-fold excess of ACV.

Fig. 3. Photoaffinity labelling of IPNS by [^{14}C]DCV

Recombinant IPNS was incubated with [^{14}C]DCV and DTT, and either kept in the dark ($-h\nu$) or irradiated ($+h\nu$) with a neodymium-YAG laser. Each sample was washed, loaded on to a Mono Q 5/5 anion-exchange f.p.i.c. column, and eluted with an NaCl gradient. The radioactive content of each fraction was determined by liquid-scintillation counting. For details see the text. IPNS protein is eluted in fractions 22–26. The peak of radioactivity eluted at 43–50 ml arises from the final wash of the column with 400 mM-NaCl, and appears not to be associated with protein. —, A_{280} ; ○—○, ^{14}C radioactivity; -----, concn. of NaCl.

This parallels the earlier results for the retention of bioactivity of IPNS when the irradiations were performed with DCV in the presence of ACV.

Thus, with [^{14}C]DCV, the selective light-dependent covalent incorporation of the modified substrate into IPNS, by using laser photolysis, has been demonstrated. Unfortunately, the low specific radioactivity of the [^{14}C]DCV made it impracticable to attempt to localize the point of insertion of the radioactive label by digestion and sequencing of ^{14}C -labelled IPNS. However, we have recently prepared tritiated DCV, with a much higher radiochemical specific activity, and are currently examining its utility in studies designed to identify the substrate binding site or sites of IPNS.

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