Inactivation of the polyketide synthase, 6-methylsalicylic acid synthase, by the specific modification of Cys-204 of the β -ketoacyl synthase by the fungal mycotoxin cerulenin

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Cerulenin, [(2*S*,3*R*)-2,3-epoxy-4-oxo-7,10-dodecadienoylamide], a mycotoxin produced by *Cephalosporium caerulens*, irreversibly inactivated 6-methylsalicylic acid synthase from *Penicillium patulum*. A combination of radiolabelling studies with [³H]-cerulenin, proteolytic and chemical digestion and N-terminal sequencing of labelled peptides indicated that the site of cerulenin modification is the highly reactive substrate-binding Cys-204 of

the β -ketoacyl synthase enzyme component. The thiol-specific inhibitor, iodoacetamide, was also shown to alkylate this residue. These findings are analogous with those observed for the reaction of cerulenin and iodoacetamide with type-I fatty acid synthases, demonstrating the close similarity between 6-methylsalicylic acid synthase and type-I fatty acid synthases.

INTRODUCTION

6-Methylsalicylic acid (6-MSA), a precursor of the mycotoxin patulin [1], is biosynthesized by *Penicillium patulum* from one molecule of acetyl-CoA and three molecules of malonyl-CoA with NADPH as a reducing cofactor. The formation of polyketide secondary metabolites, such as 6-MSA, resembles that of fatty acids both in the chemistry and nature of the multienzyme complexes involved. For instance, both polyketide synthase (PKS) enzymes and fatty acid synthases (FAS) employ repeated Claisen condensations with acyl residues, typically derived from acetyl-CoA and malonyl-CoA. In contrast with fatty acid biosynthesis, the assembly of polyketides does not always employ a complete set of keto-modifying events (reduction, dehydration and enoyl reduction) after each chain extension. During 6-MSA synthesis, for instance, β -keto reduction then dehydration occurs only after the formation of the C-6 triketide intermediate after the second condensation event [2]. 6-Methylsalicylic acid synthase (6-MSAS) bears remarkable similarities to vertebrate FAS in terms of the protein primary structure and arrangement of the enzyme activities within the multifunctional subunit [3]. In 6-MSAS the highly reactive substrate-binding cysteine thiol (Cys-204) of a β -ketoacyl synthase (KS) component from one subunit is juxtaposed with the 4'-phosphopantetheine thiol of the acyl carrier protein from an adjacent subunit. Thus formation of the condensing site in 6-MSAS requires arrangement of the subunits as functional dimers within the tetrameric complex [3].

Cerulenin [(2*S*,3*R*)-2,3-epoxy-4-oxo-7,10-dodecadienoylamide], a mycotoxin produced by the fungus *Cephalosporium caerulens*, is a potent inactivator of FAS by irreversible modification of the active cysteine thiol of the KS component [4,5]. FAS condensing enzymes from a wide variety of organisms [6], including certain isoforms of the KS from the dissociated type-II enzymes typical of plants [7] and bacteria [8,9] and the aggregated multifunctional type-I systems of fungi and animals [10] are all inactivated by this reagent. Indeed, the main exception is the FAS from *C. caerulens*, which is highly resistant to its own mycotoxin [11], presumably because of the low level of KS

component sequence homology observed when compared with FAS enzymes from cerulenin-sensitive organisms [12].

Several PKS enzymes, including 6-MSAS, have also been shown to be inactivated by cerulenin [13–16], although no evidence has been presented to indicate the site of action. Because of the apparent structural and functional similarities between PKS and FAS enzymes [17], it was proposed that, as with FAS, cerulenin inhibits the condensation step of polyketide biosynthesis [18]. In this paper, cerulenin inactivation and substrate-protection studies with 6-MSAS, in tandem with N-terminal microsequencing of peptides derived from [³H]cerulenin-labelled enzyme, are described that identify for the first time the binding site of this mycotoxin within a PKS. In comparative studies, similar investigations were performed with non-radiolabelled and ¹⁴C-labelled iodoacetamide, a reagent that has been shown specifically to alkylate the substrate-binding cysteine of vertebrate and yeast FAS enzymes [19,20].

MATERIALS AND METHODS

Materials

6-MSAS was purified from *Penicillium patulum* (NRRL 2159A), re-activated in the presence of dithiothreitol (DTT) and prepared for modification using previously described methods [3,21]. Protein concentrations were determined using Bio-Rad protein reagent (Bio-Rad Laboratories, Hemel Hempstead, Herts., U.K.). [³H]Cerulenin, radiolabelled by tritium exchange in water [8], was a gift from Dr. Penny von Wettstein-Knowles (Washington State University, WA, U.S.A.). Unlabelled cerulenin was purchased from Sigma, Poole, Dorset, U.K., and 1,3-dibromopropan-2-one (DBP) was from Lancaster Synthesis, Morecambe, Lancs., U.K. Iodo[1-¹⁴C]acetamide was purchased from Amersham International, Little Chalfont, Bucks., U.K. ProBlott PVDF membranes were obtained from Bio-Rad Laboratories and PD-10 gel filtration columns were supplied by Pharmacia LKB, St. Albans, Herts., U.K. V8 protease (endo-protease Glu-C) from *Staphylococcus aureus* was obtained from Boehringer-Mannheim, Lewes, East Sussex, U.K. Optiphase

Abbreviations used: DBP, 1,3-dibromopropan-2-one; FAS, fatty acid synthase; KS, β -ketoacyl synthase (3-oxoacyl synthase); 6-MSA, 6-methylsalicylic acid, 6-MSAS, 6-methylsalicylic acid synthase; PKS, polyketide synthase; DTT, dithiothreitol.

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HiSafe scintillation fluid was supplied by Fisons Chemicals, Loughborough, Leics., U.K. All other reagents were of analytical grade and purchased from Sigma.

Assay of 6-MSAS activity

6-MSAS activity was determined by fluorimetric measurement of the enzymic formation of 6-MSA [22]. Enzyme (0.2–1.0 m-units) was added to assay buffer containing 100 mM potassium phosphate, pH 7.6, 10 mM DTT, 1.25 mg BSA, 100 μ M acetyl-CoA and 200 μ M NADPH. 6-MSA synthesis was initiated by the addition of 200 μ M malonyl-CoA and the rate of formation of 6-MSA was measured using a Hitachi F-2000 fluorescence spectrophotometer (excitation wavelength 310 nm and emission wavelength 390 nm). Fluorescence change due to enzyme activity was related to that obtained from standard concentrations of 6-MSA. One unit of enzyme was defined as that amount catalysing the formation of 1 μ mol of 6-MSA/min at 25 °C.

Reaction of 6-MSAS with unlabelled and ^3H -labelled cerulenin

Unlabelled cerulenin was dissolved in a minimal volume of ethanol. [^3H]Cerulenin (1.67 mCi/mmol), supplied in acetone, was dried with a stream of N_2 and redissolved in a minimal volume of ethanol. 6-MSAS (typically 1–2 ml of a 1.2 mg/ml solution; approx. 1.5 μ M) in 50 mM potassium phosphate buffer, pH 7.6, containing 1 mM EDTA was incubated (30 min at 25 °C) with up to 200 μ M cerulenin. During this time, aliquots (10 μ l) were added to fluorimetric assay buffer (980 μ l) containing 2 mM DTT, so that the level of enzyme inactivation could be determined using the fluorimetric assay described above. The inactivation of 6-MSAS by cerulenin was also studied after preincubation of the enzyme for 4 min with either acetyl-CoA (500 μ M) or malonyl-CoA (500 μ M). When samples of [^3H]cerulenin-treated enzyme were required for proteolytic or chemical cleavage, unchanged reagent was removed by gel filtration through a PD-10 column that had been pre-equilibrated with fresh reaction buffer containing 2 mM DTT.

Reaction of 6-MSAS with unlabelled and ^{14}C -labelled iodoacetamide

The same procedures that had been used during investigations with cerulenin were repeated with iodoacetamide and iodo- ^{14}C acetamide (60 mCi/mmol), to allow a comparison of the reactions of cerulenin and iodoacetamide with 6-MSAS. Iodoacetamide had been shown specifically to alkylate the substrate-binding cysteine of both vertebrate [19] and yeast [20] FAS.

Binding of [^3H]cerulenin after treatment of 6-MSAS with DBP or iodoacetamide

The effect of preincubation of 6-MSAS (100 ml of a 1.5 mg/ml solution; approx. 2 μ M) with DPB (10 μ M) or iodoacetamide (100 μ M) on the binding of [^3H]cerulenin (100 μ M) was investigated. Two samples (1 ml) of enzyme in 50 mM potassium phosphate buffer, pH 7.6, containing 1 mM EDTA, were inactivated with either DBP (4 min incubation) or iodoacetamide (30 min incubation) before the addition of [^3H]cerulenin (45 min incubation). A third enzyme sample was incubated with [^3H]cerulenin alone (45 min) and a fourth control sample contained buffer only incubated with [^3H]cerulenin (45 min). After incubation, aliquots (50 μ l) of each reaction mixture were spotted on to discs of Whatman No. 1 paper (Whatman International Ltd., Maidstone, Kent, U.K.) which were immediately soaked in cold 10% (w/v) trichloroacetic acid to precipitate the 6-MSAS

protein. Unbound [^3H]cerulenin was removed by washing the disc with two changes of cold 10% trichloroacetic acid followed by one wash with cold ethanol. After the discs had been dried at 40 °C, the enzyme-bound [^3H]cerulenin was detected in Optiphase HiSafe scintillation fluid (Fisons Chemicals, Loughborough, Leics., U.K.). Radioactivity was measured using a Beckman LS 6500 counter with c.p.m. being corrected to d.p.m. using the quench corrections provided by Beckman. The average counting efficiency for ^3H was 56%.

Digestion of native and [^3H]cerulenin-modified 6-MSAS with V8 protease

To prepare peptides for separation and analysis by SDS/PAGE [21], samples of native and [^3H]cerulenin-treated 6-MSAS were digested with V8 protease (1%, w/w) at 25 °C in 100 mM potassium phosphate buffer, pH 7.6, containing 2 mM DTT. After 20 min incubations, proteolysis was terminated by the addition of an equal volume of SDS/PAGE disruption buffer [23] containing 2 mM PMSF.

Digestion of [^3H]cerulenin-treated or iodo- ^{14}C acetamide-inactivated 6-MSAS with CNBr

After incubation of 6-MSAS with [^3H]cerulenin or iodo- ^{14}C acetamide as described above, the modified protein (1 mg; 1.3 nmol) was exchanged into water using a PD-10 column and lyophilized. The residue (0.78 mg) was dissolved in 70% formic acid to a concentration of 10 mg/ml (13.3 μ M), and a 400 molar excess of solid CNBr was added. The reaction mixture was sealed under argon and placed in the dark for 24 h at room temperature. The reaction was terminated by a 5-fold dilution with water before concentration by lyophilization.

Detection of peptides derived from [^3H]cerulenin-labelled or iodo- ^{14}C acetamide-labelled 6-MSAS by fluorography

SDS/polyacrylamide gels [23] containing material from V8 protease or CNBr digests were fixed in methanol/acetic acid/water (50:10:30, by vol.) for 1 h, stained in 10% acetic acid containing 0.25% Serva Blue-G dye and destained in several changes of 10% acetic acid. Before drying under vacuum at 80 °C, gels were soaked for 30 min in the fluorographic reagent Amplify[™] (Amersham International). Autoradiography film (HyperFilm-MP[™]; Amersham International), after exposure to the gels for 4 days at –70 °C in a cassette fitted with intensifying screens, was developed and fixed according to the manufacturer's instructions.

Electroblotting of labelled peptides on to PVDF and N-terminal sequencing

Peptides found to be labelled with [^3H]cerulenin or iodo- ^{14}C acetamide were separated by SDS/PAGE and blotted electrophoretically on to PVDF membranes [24]. Peptide bands of interest were excised and sequenced by Edman degradation [25] using an Applied Biosystems 477A protein sequencer with on-line phenylthiohydantoin analysis by an Applied Biosystems 120A analyser.

RESULTS AND DISCUSSION

Inactivation of 6-MSAS with cerulenin

6-MSAS was inactivated by cerulenin in a time-dependent process as shown in Figure 1(a). The addition of a higher concentration of reagent (200 μ M) led to total inactivation within the same time course (Figure 1a). This inactivation was similar to that observed

Figure 1 Inactivation of 6-MSAS by cerulenin: substrate protection and calculation of a second-order rate constant

6-MSAS (1.4 μM) was incubated with cerulenin (100 and 200 μM) in the absence or presence of acetyl-CoA or malonyl-CoA (400 μM) (a). Gradients taken from straight line plots of log (% residual activity) versus time for various concentrations of the reagent (b) were plotted against cerulenin concentration (b, inset), from which a second-order rate constant of $13.8 \pm 0.9 \text{ M}^{-1} \cdot \text{s}^{-1}$ was calculated.

with iodoacetamide treatment of 6-MSAS [3] and FAS from yeast [26] and rat [19]. When used at a 10–140-fold molar excess, cerulenin inactivated 6-MSAS with pseudo-first-order kinetics. Linear semilogarithmic plots of percentage residual activity (x) versus time (t) for increasing cerulenin concentrations demonstrated this relationship (Figure 1b). Values of $\ln(100/x)/t$, calculated from the slopes of these semilogarithmic plots, were themselves plotted against concentration of the inhibitor (Figure 1b, inset). A second-order rate constant (k) of $13.8 \pm 0.9 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the reaction of cerulenin with 6-MSAS in 100 mM potassium phosphate, pH 7.6, was calculated from the resulting linear relationship. Fluorography of SDS/polyacrylamide gels of [^3H]-cerulenin-treated enzyme confirmed that the reagent had been incorporated into 6-MSAS (results not shown). The activity of cerulenin-treated 6-MSAS was measured before and after removal of excess reagent by gel filtration through a PD-10 pre-equilibrated with fresh buffer containing DTT. The level of cerulenin inhibition was not reversed by this treatment or by extensive dialysis, suggesting that inactivation was due to covalent binding of the inhibitor.

Effect of acetyl-CoA and malonyl-CoA on cerulenin inactivation of 6-MSAS

The starter substrate, acetyl-CoA, was found to protect 6-MSAS against inactivation by cerulenin, whereas the extender substrate, malonyl-CoA, had little protective effect (Figure 1a). This finding was analogous with that observed in previous studies on vertebrate and yeast FAS [27] using the thiol-specific reagent iodoacetamide, which was proposed to alkylate the substrate-binding cysteine residues of the KS components of these enzymes.

Reaction of 6-MSAS with iodoacetamide

The reaction of iodoacetamide with 6-MSAS, like that of

cerulenin, exhibits pseudo-first-order characteristics. The enzyme (1.4 μM) was completely inactivated by 100 mM iodoacetamide within 45 min. A second-order rate constant of $10.9 \pm 1.1 \text{ M}^{-1} \cdot \text{s}^{-1}$ was calculated (results not shown) in the same manner as for cerulenin inactivation. As had been observed with cerulenin inactivation of 6-MSAS and during the reaction of iodoacetamide with rat FAS [19], acetyl-CoA but not malonyl-CoA was found to protect 6-MSAS against iodoacetamide alkylation (results not shown). Similarly, only acetyl-CoA was found to lower significantly the level of incorporation of iodo[^{14}C]acetamide into 6-MSAS [3]. Thus it appears that cerulenin and iodoacetamide may modify the same residue within the 6-MSAS subunit.

Competitive binding of [^3H]cerulenin after DBP and iodoacetamide treatment of 6-MSAS

The homobifunctional reagent, DBP, has been shown to cross-link the substrate-binding cysteine thiol (Cys-204) of the KS and the 4'-phosphopantetheine thiol (bound at Ser-1733) from the acyl carrier protein of an adjacent subunit of 6-MSAS [3]. Iodoacetamide, a monofunctional reagent, alkylates only the substrate-binding cysteine of FAS [20] and likewise appears to alkylate Cys-204 of 6-MSAS specifically. When [^3H]cerulenin (1.67 mCi/mmol) was incubated with 6-MSAS, 2.06 nmol of [^3H]cerulenin was bound/nmol of 6-MSAS. Preincubation of 6-MSAS with DBP or iodoacetamide resulted in a lower incorporation of label, with 0.41 nmol and 0.40 nmol of [^3H]cerulenin bound per nmol of 6-MSAS respectively. Likewise, no label was detected in enzyme pretreated with non-radioactive DBP or iodoacetamide using fluorography (results not shown). Such findings, together with the substrate-protection studies for cerulenin and iodoacetamide inactivation, suggest that the primary site of both cerulenin and iodoacetamide modification in 6-MSAS is likely to be the substrate-binding cysteine (Cys-204) thiol of the KS.

Figure 2 SDS/PAGE and subsequent fluorography of peptides derived from V8 protease and CNBr cleavage of [^3H]cerulenin-labelled 6-MSAS

N-Terminal sequences of peptides containing radiolabel are indicated. The location of the N-terminal residue of each sequence, in terms of its position towards the N-terminus of the subunit from Cys-204, is also indicated. Stds., M_r standards; Autorad., autoradiograph.

Location of the site of [^3H]cerulenin modification in 6-MSAS by N-terminal sequencing of [^3H]labelled peptides after cleavage with V8 protease or CNBr

To determine unambiguously the site of modification of 6-MSAS by cerulenin, modified peptides were isolated for Edman sequencing [25]. Peptide bands containing ^3H label were identified by fluorography after SDS/PAGE of V8-protease- and CNBr-digested [^3H]cerulenin-treated 6-MSAS (Figure 2). Such bands were also identified after blotting on to PVDF and N-terminal sequencing, to allow determination of the locations of the peptides within the primary sequence [28]. Accurate M_r values for peptides were calculated using the ProtParam tool of the ExPASy World Wide Web molecular biology server, <http://expasy.hcuge.ch/sprot/protparam.html>, Geneva University Hospital and University of Geneva, Switzerland. Limits of peptides were taken from detected N-termini to residues susceptible to cleavage at positions giving M_r values similar to those estimated by SDS/PAGE.

Two major [^3H]cerulenin-labelled peptides, V8 1 and V8 2 (estimated $M_r \approx 24000$ and 22000), were detected by SDS/PAGE in lanes containing samples of V8-protease-treated enzyme. Peptide V8 1 was found to have the N-terminal sequence VASEALED, located within the proposed KS component, some 76 residues (Val-128) towards the N-terminus from Cys-204. The smaller V8 2 had the N-terminal sequence DAGIPAKS, which starts 69 residues (Asp-135) towards the N-terminus from Cys-204. Calculated M_r values for these peptides were 22804 and 22086 respectively, close to those obtained from SDS/PAGE. These results indicate that cerulenin modifies the KS component, but the large sizes of the peptides prevented identification of the precise location of cerulenin modification.

In order to determine the precise location of the modified cysteine residue, the [^3H]labelled enzyme was digested with CNBr, and labelled peptides were isolated as before. A CNBr-susceptible methionine residue (Met-194) is located close to the substrate-binding Cys-204 in the primary sequence. N-Terminal sequencing of the peptide band (CNBr 1 ; Figure 2; estimated $M_r \approx 13000$) that contained the majority ($\approx 60\%$) of the ^3H label after CNBr digestion gave the sequence GPSTAVDAAX from residue 195 to 204, with no normal residue identified from the tenth cycle of

Scheme 1 Proposed mechanism for the modification of 6-MSAS by reaction of the reagent with the substrate-binding cysteine residue (Cys-204)

Edman degradation. The Gly-195 residue is located only nine residues towards the N-terminus from Cys-204 in the KS. Sequencing of CNBr 2 (estimated $M_r \approx 18000$), a peptide that contains most of the remaining ^3H label (Figure 2), indicated the same N-terminus. As with peptides derived from V8 protease cleavage (V8 1 and V8 2), CNBr 1 (calculated M_r 11348) and CNBr 2 (calculated M_r 19356) contained Cys residues other than Cys-204, four and five respectively. However, the total conservation of Cys-204 observed on alignment of related PKS and FAS sequences indicates that this residue is the substrate-binding cysteine and thus the site of cerulenin modification.

Location of the site of iodo[^{14}C]acetamide modification in 6-MSAS by N-terminal sequencing of iodo[^{14}C]acetamide-labelled peptides after cleavage with V8 protease or CNBr

Carboxyamidomethylation of the substrate-binding cysteine residue with iodo[^{14}C]acetamide and subsequent N-terminal sequencing of the major radiolabelled species (corresponding to CNBr 1) underscored the susceptible nature of Cys-204 to alkylation. In this case the tenth cycle of Edman degradation of the iodo[^{14}C]acetamide-labelled peptide, with the experimentally detected N-terminus GPSTAVDAAX, gave a residue in which X showed a peak typical of carboxyamidomethylated Cys. Therefore, as with the reaction of cerulenin, Cys-204 was shown to be the site of iodoacetamide modification within the 6-MSAS subunit.

Conclusions

The above results indicate that 6-MSAS was inactivated with cerulenin by covalent modification of the substrate-binding cysteine thiol (Cys-204) of the KS component. Such a reaction is analogous with that observed for FAS enzymes from a variety of sources [18] and further supports the proposal that the condensing site of 6-MSAS is a functional analogue of that from type-I FAS [3]. Similarly, as found in studies with both vertebrate and yeast FAS enzymes [19,20], iodoacetamide specifically alkylates the substrate-binding cysteine thiol equivalent to Cys-204 of 6-MSAS.

The mechanism of cerulenin inactivation of 6-MSAS (Scheme 1) is proposed to be the same as that with the FAS enzymes.

Reactivity of the epoxide group of the inhibitor in protic solvents is enhanced by the formation of a lactam ring, so that, on reaction with the nucleophilic substrate-binding thiol of the KS component, the epoxide moiety is cleaved to form a covalent bond resulting in irreversible modification of the condensing activity [8,29,30]. Analogues of cerulenin that lack the epoxide ring, such as dihydrocerulenin or hexahydrocerulenin, were found to have no inhibitory effect on FAS systems [31]. The hydrophobic acyl 'tail' of cerulenin is also implicated in the inhibitory mechanism and may play a part in the recognition of the reagent by the active-site cysteine. Site-directed mutagenesis of one of a number of highly conserved glycine residues within the KS of *Saccharomyces cerevisiae* FAS to a serine residue conferred a 20–80-fold increase in cerulenin resistance on the mutant [32]. This introduction of a polar side chain may prevent the hydrophobic cerulenin molecule from binding optimally. Conserved glycine residues may form part of a hydrophobic cavity to channel substrates, fatty-acyl intermediates and fatty acid analogues such as cerulenin towards the substrate-binding cysteine residues of both FAS and PKS enzymes such as 6-MSAS.

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