Structural Similarities between 6-Methylsalicylic Acid Synthase from *Penicillium patulum* and Vertebrate Type I Fatty Acid Synthase: Evidence from Thiol Modification Studies†

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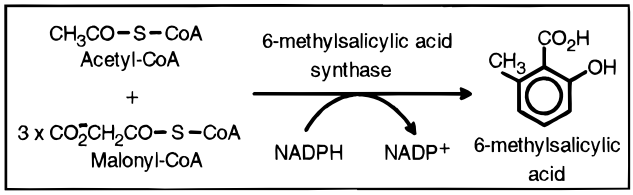
ABSTRACT: 6-Methylsalicylic acid synthase, the multifunctional enzyme complex that catalyzes the biosynthesis of the tetraketide 6-methylsalicylic acid, was modified by thiol-specific inhibitors and cross-linking reagents. Treatment with 1,3-dibromopropan-2-one caused rapid enzyme inactivation and formation of cross-linked dimers. Analysis by SDS−PAGE, density gradient ultracentrifugation, and secondary modification with [^{14}C]iodoacetamide showed that two types of cross-linked dimers were formed. Peptides derived from native and 1,3-dibromopropan[2-^{14}C]one-treated enzyme were isolated by SDS−PAGE and N-terminally sequenced. The sequences of the two N-termini from cross-linked peptides were located in the nucleotide-derived amino acid sequence and found to arise from the β-ketoacyl synthase and acyl carrier protein components of the 6-methylsalicylic acid synthase subunit. Acetyl-CoA protected the enzyme from both inactivation and cross-linking by binding to the reactive cysteine of the β-ketoacyl synthase component. Malonyl-CoA protected against cross-linking by binding to the thiol moiety of the 4′-phosphopantetheine prosthetic group of the acyl carrier protein. Formation of a mixed disulfide on treatment with 5,5′-dithiobis(2-nitrobenzoic acid) indicated that these two types of thiol residue are positioned close to each other in the active enzyme. From these studies, it was concluded that two pairs of functional dimers are present in the 6-methylsalicylic acid synthase tetramer and that, within each dimer, the β-ketoacyl synthase and acyl carrier protein components are juxtaposed to allow the respective cysteine (residue 204) and 4′-phosphopantetheine thiols to interact during condensation. This spatial arrangement of thiols at the condensing active site is analogous to that found in type I vertebrate fatty acid synthases and other polyketide synthases.

6-Methylsalicylic acid (6-MSA),[1] a precursor of the antibiotic patulin (Forrester & Gaucher, 1972), is biosynthesized by 6-methylsalicylic acid synthase (6-MSAS) in *Penicillium patulum*, from one molecule of acetyl-CoA and three molecules of malonyl-CoA with NADPH required as a reducing cofactor (Scheme 1). 6-MSA represents one of the simplest examples of the diverse group of secondary compounds known as the polyketides. Many aspects of the biosynthesis of polyketides resemble those of fatty acids in terms of both chemistry and enzymatic mechanism, despite the great variety in the polyketide structures that are formed. For instance, both syntheses employ repeated Claisen condensations for acyl chain elongation, usually involving acetyl-CoA and malonyl-CoA and, to a lesser extent, butyryl-CoA, propionyl-CoA, and methylmalonyl-CoA.

In contrast to fatty acid biosynthesis, the synthesis of polyketides does not always involve a keto-reduction step following each condensation, and it is the presence of unmodified keto groups at the alternate carbon atoms of the acyl chain that gives rise to the family name "polyketide" and offers the capacity for a variety of cyclization and chain modification events to take place. For instance, during 6-MSA synthesis, keto-reduction only occurs once after the second condensation event at the C-6 chain length (Dimroth *et al.*, 1970).

Scheme 1: Formation of 6-Methylsalicylic Acid from One Molecule of Acetyl-CoA and Three Molecules of Malonyl-CoA with NADPH as the Reducing Cofactor



The amino acid sequence of the 6-MSAS subunit and the sequential order of its enzyme activities appear to be closely related to those of vertebrate type I fatty acid synthases (FAS). 6-MSAS exists as a tetramer (α_4) of identical subunits with a native M_r value of ≈740 000 (Spencer & Jordan, 1992), while vertebrate FAS enzymes are typically homodimers (α_2) with native M_r values of ≈540 000. Using sequence alignments of 6-MSAS (Beck *et al.*, 1990), rat FAS (Amy *et al.*, 1989) and the polyketide synthase from

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[1] Abbreviations: ACP, acyl carrier protein; DBP, 1,3-dibromopropan-2-one; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; FAS, fatty acid synthase; KS, β-ketoacyl synthase; 6-MSA, 6-methylsalicylic acid; 6-MSAS, 6-methylsalicylic acid synthase; PKS, polyketide synthase; PVDF, poly(vinylidene difluoride); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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Saccharopolyspora erythraea (Bevitt *et al.*, 1992), the positions of five of the protein domains of 6-MSA have been predicted, although with varying degrees of probability. The domains for β-ketoacyl synthase (KS), acyl transferase (AT/MT), β-ketoacyl reductase (KR), and acyl carrier protein (ACP) show high levels of amino acid conservation around the putative active site residues. The substrate binding cysteine (Cys-204) of the KS and the 4′-phosphopantetheine binding serine (Ser-1733) of the ACP are invariant in sequences from all PKS and FAS systems. However, the positioning and size of the dehydratase domain (DH) are more open to question, although, in all probability, it lies between the AT/MT and KR domains. This region, within the aligned primary sequences of 6-MSAS and related enzymes, contains a conserved, active site histidine residue also found in 3-hydroxydecanoyl-thioester dehydratase from the dissociated *Escherichia coli* FAS (Cronan *et al.*, 1988).

During the synthesis of polyketides and fatty acids, the growing polyacyl chain remains covalently bound, *via* thioester links, to the enzyme systems responsible for catalysis. Two thiol residues are implicated for the covalent binding role at the condensing site: one, an active cysteine of the KS and the other from the 4′-phosphopantetheine prosthetic group of the ACP. Thiol-specific inhibitors and cross-linking reagents have been used to study the condensing sites of both the yeast and vertebrate FAS enzymes [Wakil and Stoops (1983) gives a comprehensive review]. Modification with such reagents, including 1,3-dibromopropan-2-one (DBP), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), and iodoacetamide, has provided evidence for an arrangement of the subunits to allow juxtaposition of the KS cysteine thiol and the 4′-phosphopantetheine thiol of the ACP.

The similarity between the arrangements of the functional components of 6-MSAS and FAS suggested that there are structural and functional similarities at the protein level. Preliminary experiments using the cross-linking reagent 1,3-dibromopropan-2-one (Spencer & Jordan, 1992) suggested that functional dimers are formed within the tetrameric complex. The work described in this paper has consolidated this view with evidence from a combination of thiol modification and peptide analysis that proves that the condensing site of 6-MSAS, like that of vertebrate FAS, exists as a functional dimer.

EXPERIMENTAL PROCEDURES

Materials. DBP was purchased from Lancaster Synthesis, Morecombe, Lancs, U.K., while [2-^{14}C]DBP was synthesized from [1-^{14}C]bromoacetic acid (Amersham International, Bucks, U.K.). [^{3}H]Cerulenin was a generous gift from Dr. Penny von Wettstein-Knowles, Carlsberg Research Laboratories, Copenhagen, Denmark. V8 protease (endoprotease Glu-C) was obtained from Boehringer Mannheim, Lewes, East Sussex. PD-10 gel filtration columns and Mono Q anion exchange columns were supplied by Pharmacia LKB Ltd., St. Albans, Herts, U.K. ProBlott PVDF membranes were obtained from BioRad Laboratories Ltd., Hemel Hempstead, Herts. Protein concentrations were determined using BioRad protein reagent by a modification of the method of Bradford (1976) with BSA as a standard. All other reagents were of analytical grade and purchased from Sigma Chemical Co., Poole, Dorset, U.K.

Preparation of 6-Methylsalicylic Acid Synthase for Thiol-Specific Inactivation and Cross-Linking Studies. 6-MSAS was purified from *Penicillium patulum* (NRRL 2159A) using previously described methods (Spencer & Jordan, 1992). Typically, 6 L of submerged culture (≈100 g wet weight of cells) gave rise to 8 mg of pure enzyme (sp act. 150 milliunits/mg) in a yield of 25%. Following purification, the enzyme was stored as an ammonium sulfate pellet at −70 °C, where it was stable for approximately 3 months. To ensure that the substrate binding thiol residues were fully reduced before reaction with thiol-specific inhibitors, protein pellets were resuspended in 100 mM potassium phosphate buffer, pH 7.6, containing 15% glycerol, 10 mM dithiothreitol (DTT), 2 mM benzamidine hydrochloride, and 2 mM EDTA. After 20 min incubation, the enzyme was exchanged, using a PD-10 gel filtration column, into 100 mM potassium phosphate buffer, pH 7.6, that had been thoroughly degassed. The 6-MSAS solution was either used immediately or stored for no longer than 1 h under an atmosphere of argon at 4 °C.

Preparation of 1,3-Dibromopropan[2-^{14}C]one. Synthesis of [2-^{14}C]DBP was performed essentially according to the methods of Hussain and Lowe (1968) using bromo[1-^{14}C]-acetic acid (10 μCi, ≈50 mCi/mmol). The synthesis was carried out on $^{1}/_{20}^{th}$ of the scale described and omitted the purification step at the 1-bromo-3-diazopropan[2-^{14}C]one stage as this seemed only to lower the yield. The 1-bromo-3-diazopropan[2-^{14}C]one was treated with saturated HBr, and the resulting [2-^{14}C]DBP was purified by preparative thin-layer chromatography using silica gel plates and dry benzene as the developing solvent.

Assay of 6-Methylsalicylic Acid Synthase Activity. 6-MSAS activity was determined by fluorometric measurement of the enzymatic formation of 6-MSA (Vogel & Lynen, 1975). Enzyme (0.2 −1.0 milliunit) was added to assay buffer containing 100 mM potassium phosphate, pH 7.6, 10 mM DTT, 1.25 mg of BSA, 100 μM acetyl-CoA, and 200 μM NADPH. 6-MSA synthesis was initiated by the addition of 200 μM malonyl-CoA, and the rate of its formation was measured using a Hitachi F-2000 fluorescence spectrophotometer (excitation wavelength 310 nm and emission wavelength 390 nm). The amount of fluorescence change due to the enzyme activity was related to that obtained from standard concentrations of 6-MSA. One unit of enzyme was defined as that amount catalyzing the formation of one micromole of 6-MSA per minute at 25 °C.

Inactivation of 6-Methylsalicylic Acid Synthase by Iodoacetamide. 6-MSAS (100 μL of a 1 mg/mL solution, ≈1.4 μM) was incubated at 25 °C with iodoacetamide (100 μM) in 100 mM potassium phosphate buffer, pH 7.6, for up to 30 min. During this time, aliquots (10 μL) were transferred into the fluorometric assay buffer (980 μL) described above containing 5 mM β-mercaptoethanol. Residual 6-MSA synthesis was initiated by the addition of 200 μM malonyl-CoA so that the level of enzyme inactivation due to iodoacetamide could be measured.

Samples of 6-MSAS (1 mL of a 1.4 mg/mL solution, ≈2 μM) were also incubated for 30 min at 25 °C with [1-^{14}C]-iodoacetamide (50 μM, 60 μCi/μmol), following preincubation of the enzyme in the absence and presence of its substrates acetyl-CoA (500 μM) or malonyl-CoA (500 μM). Following removal of excess inhibitor, by passage through a PD-10 column, the recovered protein was precipitated by

the addition of 0.1 mL of 50% trichloroacetic acid per 0.5 mL of protein eluate. After incubation at 0 °C for 1 h, with BSA (0.2 mg) as a coprecipitant, proteins were collected by centrifugation. Following washing of the pellets with 3 volumes (0.5 mL) of cold 10% trichloroacetic acid, the proteins were redissolved in 0.5 mL of the above buffer and assayed for bound ^{14}C-label by scintillation counting.

Reaction of 6-Methylsalicylic Acid Synthase with [^{3}H]-Cerulenin. Tritiated cerulenin (1.67 mCi/mmol), supplied in acetone, was treated with a stream of N_2 to remove the solvent, and the inhibitor residue was redissolved in a minimal volume of ethanol. 6-MSAS (typically 1−2 mL of a 1.2 mg/mL solution, ≈1.5 μM) in 50 mM potassium phosphate buffer, pH 7.6, containing 1 mM EDTA was incubated for 30 min at 25 °C with up to 200 μM cerulenin. Unreacted inhibitor was removed by gel filtration through a PD-10 column that had been preequilibrated with the same buffer containing 2 mM DTT. The level of inactivation of 6-MSAS by cerulenin was also measured (methods as above) after the enzyme had been preincubated with either acetyl-CoA (500 μM) or malonyl-CoA (500 μM) prior to exposure to the cerulenin.

Inactivation and Cross-Linking of 6-Methylsalicylic Acid Synthase with 1,3-Dibromopropan-2-one. 6-MSAS (typically 1−2 mL of a 1.2 mg/mL solution, ≈1.5 μM) was incubated with DBP (6 μM) in 100 mM potassium phosphate buffer, pH 7.6, for up to 5 min at 25 °C. Samples were prepared for fluorometric assay (as above) so that the level of enzyme inactivation due to DBP could be measured. After incubation with DBP for 5 min, a sample of 6-MSAS (30 μL) was removed for SDS−PAGE (Laemmli, 1970) to measure the extent of the cross-linking. The level of DBP inactivation and cross-linking of 6-MSAS was also measured, using the above methods, after the enzyme had been preincubated with either acetyl-CoA (500 μM) or malonyl-CoA (500 μM) prior to exposure to the DBP.

When samples of DBP-treated enzyme were required for proteolytic digestion, [2-^{14}C]DBP was used (3.6 and 8.4 μCi/mmol), and, following incubation, the enzyme was passed through a PD-10 gel filtration column that had been preequilibrated with 100 mM potassium phosphate, pH 7.6, containing 2 mM DTT to remove any unreacted reagent.

Sucrose Density Gradient Ultracentrifugation of 1,3-Dibromopropan-2-one-Treated 6-Methylsalicylic Acid Synthase in the Presence of LiCl. DBP (10 μM)-treated samples of 6-MSAS [100 μL of a 1.5 mg/ml solution (≈2 μM) in 100 mM Tris-HCl, pH 7.6, containing 2 mM DTT and 2 mM benzamidine] were analyzed by sucrose density gradient ultracentrifugation following dissociation of the complex, so that the presence of the DBP-cross-linked dimers visualized by SDS−PAGE could be confirmed. To dissociate the tetrameric complex, DBP-treated enzyme was incubated with 2 M LiCl (added as a solid) for 4 h at 0 °C (Lynen *et al.*, 1978).

Aliquots (100 μL) of DBP-treated enzyme that had been incubated with LiCl were layered onto 10 mL sucrose density gradients (10−25%, w/v) (Martin & Ames, 1960) in the above buffer containing 2 M LiCl. The samples were centrifuged in a Kontron TST 41.14 swing-out rotor (Kontron Ltd., Zurich, Switzerland) at 34 000 rpm for 8 h at 0 °C. After this time, the bases of the tubes were puctured, and 100 μL fractions were collected. Following spectrophotometric assay at 280 nm, the volume of each protein peak from the miniscus was determined. The positions of the experimental peaks were compared to those of cross-linked phosphorylase B markers (Sigma Chemical Co.) that had, similarly, been ultracentrifuged in the presence of 2 M LiCl. These contained oligomers (up to pentamer) with M_r values of multiples of the subunit M_r value of 97 400.

Protein fractions corresponding to absorbance peaks were analyzed by SDS−PAGE after the removal of LiCl by dialysis into water using Micro-Collodion bags (Sartorius, Goettingen, Germany) and concentration by lyophilization.

Inactivation and Cross-Linking of 6-Methylsalicylic Acid Synthase by 5,5′-Dithiobis(2-nitrobenzoic acid). The same procedures used for the reaction with DBP were employed for studying the reaction of DTNB with 6-MSAS. However, as the proposed cross-link induced by DTNB is likely to be a disulfide link between the substrate binding thiol residues of the KS and the ACP (Stoops & Wakil, 1982), β-mercaptoethanol (or DTT) was omitted from all buffers, and samples for SDS−PAGE were heated at 37 °C for 20 min instead of being boiled.

Digestion of 6-Methylsalicylic Acid Synthase with V8 Protease. To prepare modified peptides for separation and analysis by SDS−PAGE, samples of native and [^{14}C]DBP-treated 6-MSAS (400 μL of a 1 mg/mL solution, ≈1.4 μM) were digested with V8 protease (1% w/w) at 25 °C in 100 mM potassium phosphate buffer, pH 7.6, containing 2 mM DTT. At timed intervals, aliquots (30−50 μL) were added to 30 μL of SDS−PAGE disruption buffer (Laemmli, 1970), and the peptides produced were separated by electrophoresis using 5−15% polyacrylamide gradient gels under the conditions of Laemmli (1970). Peptides containing [^{14}C]DBP-modified thiol residues were readily identified by SDS−PAGE and autoradiography so that N-terminal sequencing could assign the positions of such peptides in the known amino acid sequence and, therefore, verify the proposed relationship between the cysteine and 4′-phosphopantetheine thiol residues in the native enzyme.

Detection of Peptides Derived from [2-^{14}C]DBP-Labeled 6-Methylsalicylic Acid Synthase by Autoradiography. To aid the identification of peptides containing [2-^{14}C]DBP-modified thiol residues, SDS−PAGE gels were subjected to autoradiography. The gels were stained in 0.1% Coomassie brilliant blue R-250 in methanol/acetic acid/water (40:7:53 v/v), destained in 3−4 changes of methanol/acetic acid/water (40:7:53 v/v), and dried under vacuum at 80 °C. Autoradiography film (HyperFilm-MP, Amersham Int.) was exposed to the gel for 2 weeks at −70 °C in a cassette fitted with intensifying screens (Kodak X-Omatic supplied by Sigma Chemical Co.). The film was developed and fixed according to the manufacturer's instructions.

Electroblotting of Peptides Separated by SDS−PAGE onto Poly(vinylidene difluoride) Membranes and N-Terminal Sequencing of Peptides. Peptides containing [2-^{14}C]DBP label were separated by SDS−PAGE and blotted onto poly(vinylidene difluoride) (PVDF) membranes. Essentially the methods used were those of LeGendre and Matsudaira, (1988). Peptides were then sequenced by Edman degradation (Edman, 1950) using an Applied Biosystems 477A protein sequencer with on-line phenylthiohydantoin analysis by an Applied Biosystems 120A analyzer. Peptides were typically sequenced to seven or eight cycles of N-terminal degradation.

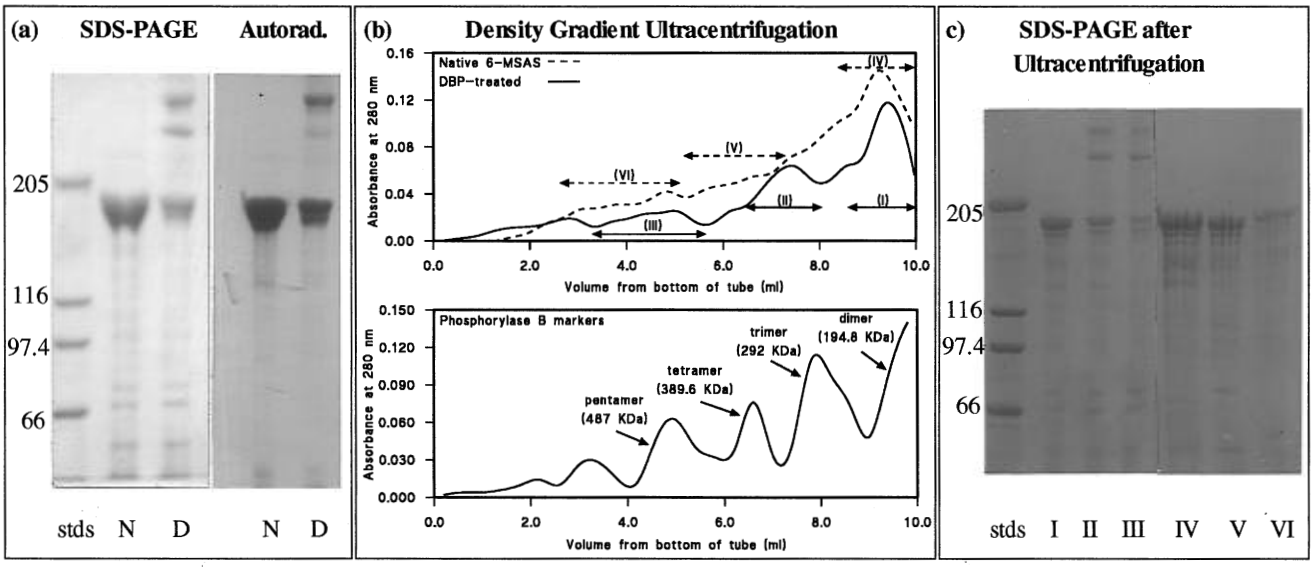

FIGURE 1: SDS−PAGE and sucrose density gradient ultracentrifugation of 6-methylsalicylic acid synthase before and after treatment with 1,3-dibromopropan-2-one (DBP) and secondary modification with [1-^{14}C]iodoacetamide. (a) SDS−PAGE gel and autoradiogram containing, in lane 1, untreated 6-MSAS and, in lane 2, 6-MSAS (1.4 μM) treated with DBP (6 μM) and secondarily modified with [1-^{14}C]iodoacetamide (50 μM) showing the incorporation of ^{14}C-label primarily into the upper band. (b) Absorbance profile following density gradient ultracentrifugation (10−25% sucrose) in the presence of 2 M LiCl (upper panel) and similar treatment of cross-linked phosphorylase B standards (lower panel). (c) SDS−PAGE analysis of pooled protein corresponding to the indicated ultracentifugation absorbance peaks (I−VI). Molecular weight standards (M_r values of 205, 116, 97.4, and 66 $\times$ 10^3) are present in those lanes marked stds.

RESULTS AND DISCUSSION

Inhibition and Cross-Linking of 6-Methylsalicylic Acid Synthase by Various Thiol-Specific Inhibitors. Determination of the nucleotide-derived amino acid sequence of 6-MSAS (Beck *et al.*, 1990) and preliminary biochemical studies on the enzyme (Dimroth *et al.*, 1970; Scott *et al.*, 1974; Spencer & Jordan, 1992) have suggested similarities to type I FAS. The monofunctional thiol-specific reagent iodoacetamide and the bifunctional thiol-specific cross-linker DBP were shown to be strong inactivating agents for vertebrate and yeast FAS enzymes (Wakil & Stoops, 1983) as was cerulenin [(2*S*,3*R*)-2,3-epoxy-4-oxo-7,10-dodecadienoylamide] from *Cephalosporium caerulens* (Vance *et al.*, 1972). DTNB was also shown to inactivate the enzyme rapidly. Since these reagents were found to react at the condensing sites of type I FAS enzymes, their effects on 6-MSAS were investigated to determine if they exhibited related effects.

(a) Iodoacetamide. 6-MSAS was inactivated in a pseudo-first-order process by a hundredfold molar excess of iodoacetamide. Complete inactivation was achieved after 30 min incubation at 25 °C in a time-dependant manner (data not shown). Enzyme that had been preincubated with malonyl-CoA (which binds to the 4′-phosphopantetheine thiol) was found to incorporate 1.97 μmol of [1-^{14}C]iodoacetamide/μmol of enzyme (0.025 μCi), a level similar to that bound when enzyme had not been pretreated with either substrate (2.09 μmol of [1-^{14}C]iodoacetamide/μmol of enzyme; 0.026 μCi). However, preincubation with acetyl-CoA (which binds to the cysteine thiol of the KS) drastically reduced this incorporation to 0.37 μmol of [1-^{14}C]iodoacetamide/μmol of enzyme (0.0065 μCi) and so indicated that, in an analogous manner to eukaryotic FAS enzymes (Kresze *et al.*, 1977; Wakil & Stoops, 1983), iodoacetamide alkylates the cysteine thiol of the KS.

(b) Cerulenin. As had been shown previously in crude extracts of 6-MSAS (Ohno *et al.*, 1972), cerulenin (200 μM) was found to inactivate the enzyme (1.4 μM) in a time-dependent manner, causing complete inactivation after 40 min (K_{obs} = 0.128 min^{-1}). Enzyme activity was not restored after removal of excess reagent by gel filtration through a PD-10 column or by the addition of DTT (data not shown). Addition of acetyl-CoA (500 μM), prior to incubation with cerulenin, was found to protect the enzyme from inactivation (K_{obs} = 0.046 min^{-1}) whereas addition of malonyl-CoA (500 μM) had very little protective effect (K_{obs} = 0.115 min^{-1}). Thus, in 6-MSAS the site of reaction of the epoxide moiety of cerulenin would appear to be the cysteine thiol of the KS component, as has been shown with FAS from a variety of sources (Kauppinen *et al.*, 1988; Vance *et al.*, 1972).

(c) 1,3-Dibromopropan-2-one. (i) Inactivation and Cross-Linking of 6-Methylsalicylic Acid Synthase. The bifunctional reagent, DBP, when used in a quantity stoichiometric to the proposed number of condensing sites, *i.e.*, four per tetrameric complex (Spencer & Jordan, 1992), was found to cause a very rapid inactivation of 6-MSAS, with a $t_{1/2}$ of about 7 s. When DBP-treated samples of 6-MSAS were analyzed by SDS−PAGE, bands of lower mobility (higher M_r) were observed (Figure 1a), suggesting that cross-linking had occurred. Typically, two bands of higher M_r were visualized. The molecular mass standards used had a maximum M_r value of 205 000 (myosin), and although extrapolation of the standard curve constructed from them yielded apparent M_r values of 320 000 and 360 000, the density gradient ultracentrifugation methods described below were employed to confirm the proposed dimeric nature of these cross-linked species.

(ii) Density Gradient Ultracentrifugation of Cross-Linked 6-Methylsalicylic Acid Synthase. Density gradient ultracentrifugation of DBP-treated enzyme in the presence of the disassociating agent (2 M LiCl) with subsequent spectrophotometric analysis and SDS−PAGE of peak fractions confirmed that both the proposed cross-linked bands visualized by SDS−PAGE were indeed dimeric in nature. Fol-

lowing separation of the subunit monomers and cross-linked dimers by ultracentrifugation (Figure 1b), removal of LiCl, and concentration of fractions corresponding to the protein peaks, SDS−PAGE was used to reanalyze the protein bands to allow characterization of the postultracentrifugation material (Figure 1c). When analyzed by SDS−PAGE, proteins corresponding to the ultracentrifugation peaks (marked II and III on Figure 1b) were found to contain cross-linked subunit species, as well as some bands of native subunit M_r value. Non-cross-linked subunits may be present in these lanes due either to overlapping of the different subunit species during collection or to incomplete disassociation of the complex during LiCl incubation. The protein peak marked I on Figure 1b was found, upon SDS−PAGE, to contain the 6-MSAS subunit monomer only, and so verified the position of this $M_r \approx 180\,000$ species within the gradient profile. Similarly, peaks labeled IV−VI in Figure 1c, derived from non-DBP-treated enzyme, were all found to contain the monomeric subunit, indicating again the incomplete nature of the LiCl dissociation. The experimental protein peaks containing the cross-linked bands correspond closely to those of the similarly treated cross-linked phosphorylase B markers. The tetramer peak of the phosphorylase B markers (M_r of 389 600) sedimented close to the protein peaks containing the two types of cross-linked subunits of 6-MSAS (Figure 1b). In addition, the fact that both cross-linked bands were located within the same ultracentrifugation peak indicates strongly that they have approximately the same M_r value, despite their different mobilities on SDS−PAGE.

The presence of two DBP-derived bands with molecular masses close to the value expected for a cross-linked dimer (total $M_r \approx 360\,000$) eliminates the possibility that such bands are cross-linked trimers or tetramers and may be explained by different levels of cross-linking within the proposed dimer. For instance, subunits cross-linked at both of the condensing sites in a proposed dimer would be expected to electrophorese faster than dimers cross-linked at only one of the two condensing sites, as the double-cross-linked species would be likely to maintain a more compact structure in the presence of SDS.

(iii) Secondary Modification with [^{14}C]Iodoacetamide. The probablity that both of the DBP bands visualized by SDS−PAGE were dimeric in nature was supported by the finding that when [^{14}C]iodoacetamide was incubated with the enzyme (50 μM, 20 min), after the addition of DBP and subjection of the SDS−PAGE gels of the samples to fluorography, the upper band of the DBP-induced dimers (the band proposed to be singly cross-linked) contained the vast majority of the cysteine thiol-specific [^{14}C]iodoacetamide label (Figure 1a). It is interesting to note the higher resolution of the separation of such differently cross-linked bands by SDS−PAGE compared with techniques such as ultracentrifugation, presumably due to the sieving effect of the polyacrylamide gel and the effect of cross-links between the subunits in the presence of SDS.

(iv) Radiolabeling with Dibromopropan[2-^{14}C]-one. When [2-^{14}C]DBP was added to 6-MSAS and the enzyme was analyzed by SDS−PAGE and autoradiography, both bands of cross-linked protein were found to contain the radiolabel as was the residual band left at the monomeric M_r of ≈180 000. Thus, it appeared that radiolabeled DBP was incorporated into 6-MSAS, causing cross-linking of the subunits and labeling of single subunits by alkylation of the reactive cysteine thiol alone, as suggested by the following substrate protection studies.

(v) Substrate Protection against the Reaction with Dibromopropan-2-one. As with cerulenin, preincubation with acetyl-CoA (500 μM) prior to addition of DBP was found to protect the enzyme from inactivation ($K_{obs} = 0.41$ min^{-1}), whereas addition of malonyl-CoA had no significant protective effect ($K_{obs} = 2.7$ min^{-1}), yielding a level of inactivation similar to that of non-pre-treated enzyme ($K_{obs} = 3.2$ min^{-1}). However, the addition of either acetyl-CoA or malonyl-CoA prior to incubation with DBP was found to prevent almost all the cross-linking (data not shown; Spencer & Jordan, 1992). Likewise, preincubation of the enzyme with iodoacetamide prior to addition of DBP also prevented cross-linking. It would appear that acetyl-CoA is able to protect both the cysteine and 4′-phosphopantetheine thiol groups from the effects of DBP, whereas malonyl-CoA can only protect against cross-linking, but cannot prevent the reaction of the reagent with the 4′-phosphopantetheine thiol causing enzyme inactivation. It is likely that acetyl-CoA protects the highly reactive cysteine thiol group of the KS against DBP reaction and that malonyl-CoA binds to and protects the 4′-phosphopantetheine thiol of the ACP. The remote possibility that the protection afforded by acetyl-CoA and malonyl-CoA was an artifactual result, due to the reaction of the acyl-CoA thioester or residual CoA with DBP, was eliminated by analyzing control reaction mixtures of acetyl-CoA and DBP or malonyl-CoA and DBP using electrospray mass spectrometry (negative ion). No evidence for adduct formation was observed. During cross-linking, DBP is likely to react initially with the more reactive KS component (based on the strong reactivity of this thiol alone with other reagents and the protective effects of the substrates), allowing reaction of the other functional group of the alkylating reagent with the 4′-phosphopantetheine thiol of the adjacent subunit. As with vertebrate FAS, it is proposed that to allow condensation to proceed during 6-MSA biosynthesis, the cysteine thiol of one subunit must be able to become closely juxtaposed to the 4′-phosphopantetheine thiol of an adjacent subunit.

(d) 5,5′-Dithiobis(2-nitrobenzoic acid). The inactivation and substrate protection studies for the reaction with DBP indicated that the subunits of 6-MSAS had become cross-linked due to the juxtaposition of the substrate binding thiol residues. Close positioning of these sulfhydryl groups was predicted to allow formation of a mixed disulfide during DTNB treatment, as had been found with chicken liver FAS (Stoops & Wakil, 1982). Treatment of 6-MSAS (1.4 μM) with DTNB (10 μM) resulted in a rapid loss of activity ($t_{1/2} \approx 15$ s, $K_{obs} = 2.76$ min^{-1}). Protection against inactivation was afforded by both acetyl-CoA ($K_{obs} = 0.34$ min^{-1}) and malonyl-CoA ($K_{obs} = 0.48$ min^{-1}), indicating that the 4′-phosphopantetheine thiol group may be able to bind DTNB directly, in apparent contrast to the reaction with DBP. SDS−PAGE of such samples, performed in the absence of β-mercaptoethanol to maintain the disulfide bonds, gave rise to cross-linked bands, similar to those seen with DBP (data not shown). These results suggested that DTNB was able to cross-link the juxtaposed thiols of adjacent subunits by a direct disulfide link. On this basis, the thiol groups are likely to be positioned within approximately 2 Å of each other, as proposed for FAS by Stoops and Wakil (1982). The disulfide cross-link was found to be broken by the addition

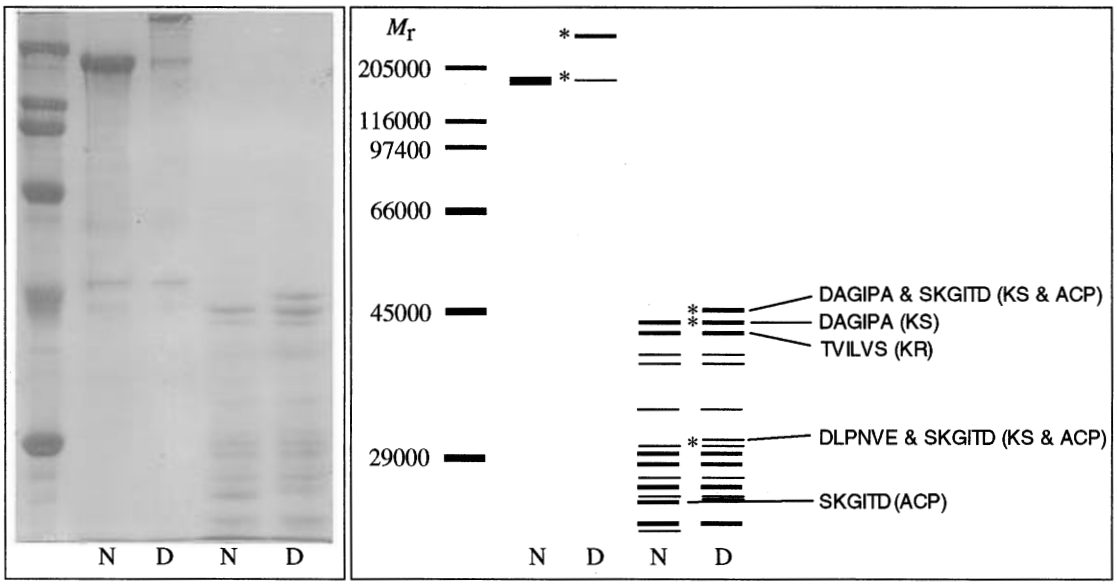

FIGURE 2: SDS−PAGE of native and 1,3-dibromopropan[2-^{14}C]one-treated 6-methylsalicylic acid synthase following V8 protease digestion. V8 protease-derived peptides from both nontreated (N) and [^{14}C]DBP-treated enzyme (D) were separated alongside each other in 5−15% polyacrylamide gradient gels. N-Terminal sequences of peptides exposed to Edman degradation are indicated on the representation of the gel, as are those peptide bands found to contain radiolabel (*).

of 10 mM β-mercaptoethanol, resulting in a recovery of 60% of the original activity within 20 min incubation at room temperature and restoration of the monomeric protein band on SDS−PAGE (data not shown).

Isolation of Peptides Derived from Native and [2-^{14}C]-1,3-Dibromopropan-2-one-Treated 6-Methylsalicylic Acid Synthase. When peptides resulting from limited V8 protease treatment (1%) on samples of native and DBP-treated 6-MSAS were analyzed by SDS−PAGE, discrete differences in the protein bands of the different samples were found. Figure 2 shows peptide bands resulting from SDS−PAGE of such samples. In those lanes containing peptides from DBP-modified enzyme (D), bands with M_r values of ≈36 000 and 40 000, not observed in nontreated samples (N), were routinely detected. Similarly, in the native samples (N), protein bands (M_r value of ≈17 000) were found that were not present in DBP-modified samples (D). It is likely that the new bands seen in the DBP-treated samples (D) were due to cross-linking between the protein subunits. In view of the previous observations on the action of DBP on 6-MSAS (Spencer & Jordan, 1992) and FAS (Wakil & Stoops, 1983), it was predicted that the new bands contained peptide chains from both the KS and ACP regions (in which the active thiols are cross-linked by the DBP). This cross-linking explains the occurrence of certain peptide bands in native samples (N) only as they are able to become cross-linked to other peptide fragments *via* DBP to form those bands seen only in DBP samples (D).

To identify the cross-linked peptides unambiguously, [2-^{14}C]DBP was used to modify the enzyme to allow detection of peptides containing the 2-^{14}C-radiolabel by autoradiography (Figure 2). No other bands were found to be radiolabeled under the conditions used. Putative cross-linked peptides, found only in DBP-treated enzyme samples, were shown to contain the 2-^{14}C-label. In addition, some bands (M_r range of 30 000−40 000), found in both DBP-modified (D) and native samples (N), were found to be ^{14}C-labeled.

Table 1: N-Terminal Sequences Detected from V8 Protease-Derived Peptides of Native and 1,3-Dibromopropan[2-^{14}C]one-Treated 6-Methylsalicylic Acid Synthase (6-MSAS)[a]

peptide band name	6-MSAS source	apparent M_r value	N-termini detected and residue yields (pmol)
DBP[1]	X-link	40000	$D_{10}A_9G_{16}I_{15}P_8A_9$ and $S_4K_5G_{16}I_{15}T_5D_7$
DBP[2]	native	38000	$D_5A_4G_5I_4P_4A_4$
DBP[3]	X-link	36000	$D_4L_3P_3N_4V_3E_4A_5$ and $S_3K_3G_2I_2T_3D_4V_3$
DBP[4]	X-link	29000	$D_2L_1P_2N_1V_1E_2$ and $S_2K_1G_2I_1T_1D_1$
Nat[1]	native	17000	$S_7K_{10}G_{16}I_{18}T_{17}D_{15}$

[a] The N-terminal sequences were located within the 6-MSAS subunit by inspection of the nucleotide-derived amino acid sequence (Beck *et al.*, 1990). It should be noted that residue yields given for the shared amino acids G and I at positions 3 and 4 in both sequences of DBP[1] are totals for that cycle of Edman degradation.

Sequencing of 2-^{14}C-Labeled and Cross-Linked Peptides. Selected protein bands, identified as being modified by DBP by the incorporation of the ^{14}C-label, were subjected to N-terminal sequencing after electroblotting onto PVDF membranes. Proposed cross-linked species gave rise to two amino acids for each cycle of the Edman degradation, as expected, since there would be an N-terminal residue detected for each of the cross-linked peptides. Pure peptides from non-cross-linked protein gave one sequence. Experimentally derived sequences (shown in Table 1) were located in the derived amino acid sequence (Beck *et al.*, 1990) as shown in Figure 3 and illustrated in Figure 2.

N-Terminal sequencing of putative cross-linked peptides, the 2-^{14}C-labeled peptide present in both native (N) and DBP-treated samples (D) and a peptide found only in material derived from native enzyme were performed. A peptide with a M_r value of ≈40 000 (DBP[1] in Table 1 and Figure 3) revealed N-terminal sequences DAGIPA and SKGITD, that are located some 102 residues into the putative KS activity (Bevitt *et al.*, 1992) and 80 residues before the proposed start of the ACP region, respectively. The same DAGIPA

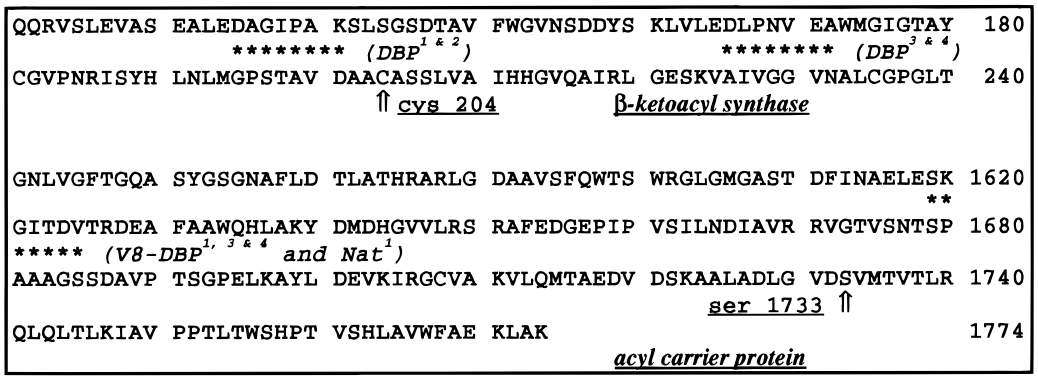

FIGURE 3: Nucleotide-derived amino acid sequences from portions of the β-ketoacyl synthase and ACP regions of the 6-methylsalicylic acid synthase subunit showing the locations of experimentally derived N-terminal sequences. Indicated (upward arrows) are the active cysteine (Cys 204) and 4′-phosphopantetheine binding serine residues (1733) and the positions of the N-terminal sequences detected from peptides derived from native and 1,3-dibromopropan[2-^{14}C]one-treated enzyme (asterisks). The amino acid residue numbers shown are the number of residues from the N-terminal methionine (Beck *et al*., 1990).

sequence of the KS component was obtained for a peptide with a M_r value of ≈38 000 (DBP^2 in Table 1 and Figure 3) found in both native and DBP-treated enzyme digests. When 6-MSAS had been treated with [2-^{14}C]DBP prior to digestion, this peptide from the KS was shown to contain radiolabel. The radiolabeling of this peptide (M_r value of ≈38 000), that results from the reaction of DBP with Cys-204 of the KS activity, highlights the unusually high reactivity of this residue toward alkylating agents. Alkylation with DBP of the 4′-phosphopantetheine thiol alone did not appear to occur, suggesting that the reagent must react in an ordered fashion, with the KS cysteine thiol being modified first. Thus, the 4′-phosphopantetheine thiol was only alkylated by DBP in cross-linked enzyme preparations as judged by the absence of a ^{14}C-labeled peptide with only the N-terminal sequence SKGITD.

Other putative cross-linked peptide bands with M_r values of ≈36 000 (DBP^3 in Table 1 and Figure 3) and ≈29 000 (DBP^4 in Table 1 and Figure 3), that occur only in DBP-modified samples, gave rise to N-termini of DLPNVE and SKGITD. The location of the former sequence was found to be 38 residues toward the N-terminus from Cys-204, with the latter sequence being the same ACP-containing portion as was found with DBP^1. Since the peptides were only isolated from cross-linked dimers and gave the sequences DLPNVE and SKGITD (from the KS and a peptide portion containing the ACP component, respectively), the results heavily support the proposal that the Cys-204 and 4′-phosphopantetheine thiols of these regions are indeed juxtaposed to allow cross-linking with DBP to occur. Although there are other, unconserved cysteines within the KS peptide portions recovered, the total conservation of Cys-204 among related PKS and FAS sequences indicates that this residue forms the condensing enzyme active site together with the 4′-phosphopantetheine thiol of the ACP. To indicate the positions of these sequences relative to the residues of interest, Figure 3 shows nucleotide-derived amino acid sequences of the 6-MSAS subunit from regions containing the substrate binding cysteine and the 4′-phosphopantetheine serine along with the detected N-terminal sequences.

A peptide with a M_r value of 17 000 (Nat^1 in Table 1 and Figure 3) that occurs only in samples derived from native enzyme was found to have the now familiar SKGITD sequence. The SDS−PAGE estimated M_r value of ≈17 000 and the detected N-terminus indicate that this peptide includes the whole of the predicted ACP region (Bevitt *et al.*, 1992) and stretches to the extreme C-terminal lysine residue (Lys-1774) of the subunit (or the last susceptible Glu residue before it). Upon reaction with DBP, this "ACP peptide" with its 4′-phosphopantetheine prosthetic group (bound at Ser-1733) becomes cross-linked to Cys-204 of the KS component. This explains the occurence of the M_r ≈17 000 peptide band only in samples from native enzyme and the appearance of additional bands in the appropriate gel lanes when the enzyme was incubated with DBP.

CONCLUSIONS

The studies described in this paper support the proposal that the condensing site of 6-MSAS is structurally and functionally analogous to type I fatty acid synthases. Cross-linking of the 6-MSAS subunits with DBP and DTNB takes place between the cysteine SH of the KS of one subunit and the 4′-phosphopantetheine thiol of an adjacent subunit, leading to the "trapping" of dimers. N-Terminal sequencing of peptides derived from DBP-treated 6-MSAS provides unambiguous evidence for the first time that KS and ACP thiols from separate subunits are located close to each other at the condensing site. The proximity of the N-termini detected to the substrate binding cysteine (Cys-204) and 4′-phosphopantetheine binding serine (Ser-1733) residues, coupled with the total conservation of these residues in PKS and FAS enzymes, confirms the arrangement and role of these thiol residues at the condensing sites. Such findings support earlier suggestions that the subunits of type I FAS (Wakil & Stoops, 1983) and PKS (Spencer & Jordan, 1992) systems exist as functional dimers. Whereas Wakil and Stoops (1983) interpreted their results to suggest that the FAS forms "head to tail" dimers, the results presented in this paper also correlate with a recent PKS model (Staunton *et al.*, 1996) in which the 6-DEBS proteins form "head to head" homodimers with a double-helical structure. A similar "head to head" model for 6-MSAS would also allow direct interaction of the KS and ACP from adjacent subunits.

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