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Biosynthesis of the Immunosuppressant Immunomycin: The Enzymology of Pipecolate Incorporation†

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ABSTRACT: Immunomycin, an immunosuppressant closely related to FK 506, contains a pipecolate residue in amide linkage with an acyl group in its polyketide backbone. An enzyme activating L-pipecolic acid has been isolated from *Streptomyces hygroscopicus var. ascomyceticus*, which produces immunomycin. Purification results in a monomer of 170 kDa exhibiting N-terminal heterogeneity, apparently arising from proteolysis of a single species. It is a dimer under native conditions. The reaction appears to use an aminoacyl adenylate as an intermediate in the activating reaction, as do most activating enzymes involved in nonribosomal peptide synthesis. A range of pipecolate and proline analogues act as substrates in the pyrophosphate–ATP exchange resulting from the adenylation reaction. Several analogues are inhibitors of the subsequent thioesterification of the enzyme. Antibody raised to the purified enzyme was used to follow antigen during the course of fermentation. Maximal levels of antigen are found when synthesis of immunomycin is maximal. Ten of twelve immunomycin nonproducing mutants lack detectable pipecolate-activating enzyme in Western blots. From the enzymatic characteristics, substrate specificity, and immunological properties, we propose that we have isolated the enzyme responsible for activating pipecolic acid for immunomycin biosynthesis.

Immunomycin, first isolated under the name ascomycin (Arai et al., 1962; Hatanaka et al., 1988b) and subsequently named FR-900520 or FK520, is a peptidolactone produced by *Streptomyces hygroscopicus* strains. It is structurally related to FK 506, the potent immunosuppressant produced by *Streptomyces tsukabiensis* (Kino et al., 1987; Hatanaka et al., 1988b) differing only at one position of the polyketide chain (Figure 1). A four-carbon unit of the polyketide region of immunomycin originates from the incorporation of a butyrate residue while the corresponding five-carbon chain arises in FK 506 from the condensation of a propionate with an acetate residue (Byrne et al., 1991). Each product consists of a lengthy polyketide region bridging a substituted cyclic seven-carbon unit and an amino acid, pipecolic acid, or proline, via a lactone bond involving the carboxyl of the amino acid (Figure 1). In *S. tsukabiensis* the pipecolate-containing component is the principal product, with proline incorporation occurring only to a minor degree. The proline analogue (Hatanaka et al., 1988b) is considerably less potent in immunosuppression, implying that the amino acid portion of the structure is important for bioactivity. Since the amino acid is in amide linkage with an acyl group of the polyketide, it is reasonable to speculate that it is activated in the manner described for the nonribosomally synthesized peptide antibiotics (Kleinkauf & von

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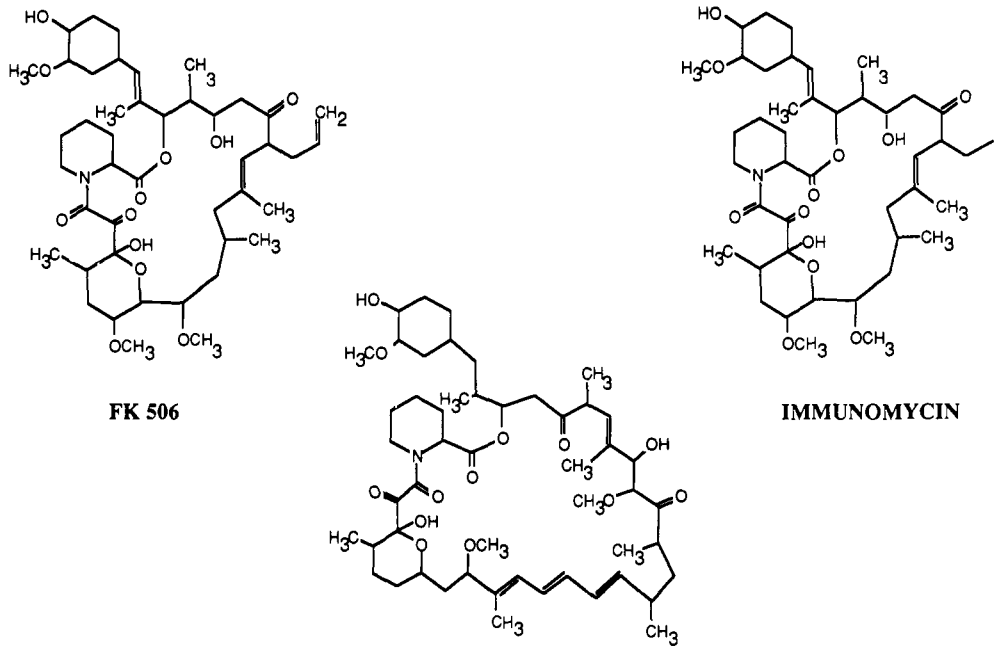

FIGURE 1: The structures of FK 506, immunomycin, and rapamycin.

Dohren, 1987). This indeed was proposed, without any experimental evidence, for the insertion of the pipecolic acid residue in a closely related structure, rapamycin (Figure 1) (Paiva, 1988). The enzymology of amino acid activation and nonribosomal peptide bond formation has received considerable attention (Kleinkauf & von Dohren, 1987) since it was first described by Lipmann (Lee & Lipmann, 1975). Briefly, the amino acid is activated by ATP, generally by the formation of an acyl adenylate, although there is evidence for aminoacyl phosphate formation in a few cases (Kleinkauf & von Dohren, 1987). Activation is followed by transfer to a cysteine residue on the enzyme to form the amino acid thioester. Peptide bond formation occurs after transfer of the aminoacyl residue to a phosphopantetheine arm on a second enzyme component of the peptide synthetase. In a series of papers on peptides and peptidolactones (Keller et al., 1984; Keller, 1987; Glund et al., 1990), Keller has employed to great advantage a property shared by many such activating enzymes, namely, the formation of amino acid adenylates as intermediates in the activation reaction. Consequently, these enzymes catalyze an amino acid dependent ATP-exchange reaction. Furthermore, they catalyze the rapid binding of the amino acid to the enzyme in thioester linkage, independent of peptide bond formation. Both of these reactions constitute facile assays of this class of activating enzymes in the complete absence of information about the succeeding reaction, which is often the case in complex molecules like immunomycin. Both assays may be used for purification of the activating enzyme and characterization of amino acid specificity. Using this approach, we have isolated and characterized an enzyme activating pipecolic acid in immunomycin-producing strains.

Experimental Procedures

Strains and Media. Enzyme was isolated from ATCC 55087, an improved producer derived from *S. hygroscopicus var. ascomyceticus* (ATCC 14891). Both parent and its derivative produce predominantly immunomycin and small amounts of FK 523, the analogue with a propyl unit replacing the butyryl unit in the polyketide portion (Hatanaka et al., 1988a). Strains tested for immunologically cross-reacting protein were *S. tsukabiensis 9993*, the FK506 producer, and *S. hygroscopicus* NRRL 5491, a rapamycin producer. Strains were stored as lyophilized cultures or as frozen vegetative mycelia in seed medium containing 5% glycerol. Seed medium contains (in grams per liter) Hycase SF (Sheffield), 20; yeast extract (Difco), 20; glucose, 20; NaCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.025; $MnSO_4 \cdot H_2O$, 0.005, $ZnSO_4 \cdot 7H_2O$, 0.01; $CaCl_2 \cdot 2H_2O$, 0.02; and KNO_3, 2, adjusted to pH 7.0 before autoclaving. Inoculum grown in 25 mL of seed per 250-mL baffled Erlenmeyer flask for 44–48 h at 27 °C and 220 rpm was inoculated at 3% into 30 mL in a 250-mL Erlenmeyer flask of production medium, containing (in grams per liter) glucose, 22; $CaCO_3$, 0.25; lactic acid, 1.7; yeast extract, 15; MOPS buffer, 10; glycerol, 25; L-tyrosine, 4; and trace elements, 5 mL/L (stock contains (in grams per liter in 0.6 N HCl) $MgSO_4 \cdot 7H_2O$, 61.1; $CaCO_3 2$; $FeCl_3 \cdot 6H_2O$, 5.4; $ZnSO_4 \cdot 7H_2O$, 1.44; $MnSO_4 \cdot H_2O$, 1.11; $CuSO_4 \cdot 5H_2O$, 0.25; H_3BO_3, 0.062; $Na_2MoO_4 \cdot 2H_2O$, 0.49; $CoCl_2 \cdot 6H_2O$, 0.28), adjusted to pH 6.8 before sterilization. Incubation was carried out at 27 °C, 240 rpm for 64–68 h.

Materials and Methods

[*carboxyl*-^{14}C]-DL-Pipecolic acid (7.1 mCi/mmol) was prepared by the Drug Metabolism Department at Merck and repurified by preparative TLC (butanol/acetic acid/water 60:15:15 on silica plates) before use. [*ring*-U-^{3}H]Pipecolic acid (3.1 mCi/μmol) was prepared by Dr. Avery Rosegay of Merck and Co.

Enzyme Assay. PP–ATP exchange was measured by a modification of Lipmann's method (Lee & Lipmann, 1975); 2.5 mM ATP, 4 mM $MgSO_4$, 0.5 mM sodium pyrophosphate, 0.1 mM EDTA, 1 mM DTT, 75 mM HEPES, pH 7.3, 30000 dpm [^{32}P]pyrophosphate, 2–5 μL of enzyme, and 10 mM L-pipecolic acid were incubated in a final volume of 50 μL for 10 min at 30 °C. The reaction was terminated by the addition of 0.3 mL of a 1% suspension of activated charcoal (Merck) in 3% perchloric acid and 10 mM sodium pyrophosphate. The charcoal was collected by filtration through glass-fiber filters, washed with 6 mL of cold water, and counted in 5 mL of Scintiverse (Fisher).

Amino acid binding was measured by a modification of Keller's method (Keller, 1987); 1 mM ATP, 15 mM $MgSO_4$,

1 mM DTT, 75 mM HEPES, pH 7.3, 0.25 μCi DL-pipecolate, and enzyme (up to 80 μL) were incubated in a final volume of 100 μL for 10 min at 30 °C. The reaction was terminated by the addition of 1 mL of 7% trichloroacetic acid containing 1 mM pipecolate and sufficient carrier bovine serum albumin to bring the total protein to 100 μg. After 30 min at 4 °C, the pellets were harvested by microfuge centrifugation and washed three times with cold trichloroacetic acid plus pipecolate and twice with ethanol. The final pellet was dissolved in 50 μL of 100% formic acid for counting in 5 mL of Scintiverse at about 90% efficiency. Units are expressed as picomoles or nanomoles of pipecolate bound under these conditions. For examination of thioester properties, formic and performic acid treatment of pellets followed by thin-layer chromatography on silica with butanol/acetic acid/water 60:15:15 were performed essentially as described by Keller (Keller, 1987). Radioactivity was detected by a Bioscan System 200 imaging scanner at about 6% efficiency for ^{14}C.

Enzyme Extraction and Isolation. Mycelia harvested after 40 h of incubation in production medium were washed three times in 0.5 M KCl to remove extracellular and loosely bound proteins. Fifty grams of freshly packed mycelia from 600 mL of culture was resuspended in 3 volumes of extraction buffer containing 0.025 M HEPES, pH 7.3, 20% glycerol, 0.5 mM EDTA, 0.2 mM dithiothreitol (DTT), 0.5% Triton X-100, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), and 0.1 mM diisopropyl fluoride and disrupted by sonication at 4 °C for a total of 5 min in 1-min bursts interrupted by cooling intervals. All procedures throughout purification were performed at 4 °C. The pellet after centrifugation at 20000*g* for 15 min was reextracted by sonication twice more in 1 volume of extraction buffer. The combined extracts were brought to 30% saturation with ammonium sulfate, the small pellet was discarded, and then the extracts were brought to 65% saturation and centrifuged. The pellet was stored overnight at 4 °C without desalting. It was dissolved in purification buffer containing 0.025 M HEPES pH 7.3, 10% glycerol, 0.5 mM EDTA, 0.2 mM DTT, 0.1 mM PMSF and dialyzed against frequent changes of this buffer for about 4 h to reduce the conductivity to below 10 mS. The desalted extract was then applied to microgranular DEAE-cellulose (300 mL for 60 g of mycelium) in purification buffer and eluted at about 0.3 M KCl applied as a linear gradient from 0 to 0.6 M, with a flow rate of about 40 mL/h.

The broad peak located by pipecolate-dependent PP–ATP exchange was concentrated by precipitation with 70% ammonium sulfate. The pellet was washed twice with 55% ammonium sulfate before being dissolved in a minimum volume of purification buffer containing 0.1 M KCl and applied to a Sephacryl S300 column (2-cm i.d. × 100 cm). Elution was performed in purification buffer containing 100 mM KCl overnight at a flow rate of about 20 mL/h. The broad peak eluting with the bulk of the included protein was concentrated by centrifugation in Centriflo cones CF25 (Amicon) and applied to a MonoQ HR 10/10 ion-exchange column. Elution was achieved by a shallow KCl gradient between 0.18 and 0.25 M, with a flow rate of 1.5 mL/min over a period of 2 h. Active fractions were pooled and concentrated with Centriflo cones. At this point the enzyme is stable if stored in 20% glycerol at −20 °C.

Electrophoresis. Preparative SDS electrophoresis was performed on 1.5-mm-thick 6% Laemmli gels. Elution of the enzyme from the gel was performed after brief staining in 0.1% Coomassie blue in 50% methanol by agitating cut gel pieces gently overnight in 0.1% SDS. One-millimeter-thick 8% SDS gels from Novex (Encinatas, CA) were used for analytical purposes because they show greater resolution of the bands around 170 kDa. The standards used were a high molecular mass mixture from Pharmacia and *Escherichia coli* RNA polymerase containing the β and β′ subunits at 155 and 165 kDa (Pierce). Blotting onto PVDF paper (Bio-Rad) was performed in a Bio-Rad Trans Blot cell in 0.015 M Tris/0.09 M glycine, pH 8.3, and 8 V overnight. Protease mapping (Cleveland et al., 1977) was performed as with V8 protease from Miles. The isoelectric point was determined on Phast (Pharmacia) isoelectric focusing gels, pH 3–9 and 4.5–6.

Molecular Mass Determination. Native molecular mass was determined by gel filtration on Superose 12 (Pharmacia) and TSK-SWP (7.5 × 750 mm) in 50 mM HEPES, pH 7.3/150 mM KCl/0.5 mM DTT at 4 °C, after calibration of each with bovine thyroglobulin (600 kDa), ferritin (450 kDa), catalase (240 kDa), aldolase (160 kDa), and bovine serum albumin (67 kDa). Phosphate buffer, which minimizes the interaction of standards with the resin bed (especially important for aldolase, which is a particularly useful standard, having a native size of 160 kDa), causes loss of pipecolate-activating enzyme activity and could not be used. The addition of at least 100 mM KCl gave nearly linear calibration curves on the two sizes of columns employed.

Antibody Preparation. The family of bands migrating at about 170 kDa in 6% gels was cut after brief staining in 0.1% Coomassie blue in 50% methanol. The gel was macerated; the protein was eluted overnight at 30 °C into 0.1% SDS with gentle shaking and concentrated by lyophilization. Antibody was produced in rabbits following the popliteal lymph node procedure (Sigel et al., 1983) with slight modifications. Briefly, a total of 20 μg in complete Freund's adjuvant was administered in a divided dose into each popliteal lymph node in the hind legs, following surgical incision. Secondary injections in incomplete Freund's adjuvant were given subcutaneously in multiple sites on the shaved backs, and serum was taken 10–14 days following each booster inoculation. The resulting antiserum was used at 1:10000 dilution in Western blotting, with use of goat anti-rabbit antiserum coupled to alkaline phosphatase as the detection system and 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Promega) as substrate. Developed Western blots were quantitated after the PVDF membrane was dried in a Shimadzu Scanning Spectrophotometer CS 920 at 560 nm.

Pipecolate Adenylate Synthesis. Pipecolate adenylate was synthesized by use of dicyclohexylcarbodiimide (Berg, 1958) essentially as described by Keller (Keller et al., 1984). It could not be distinguished from AMP by TLC on silica plates in butanol/acetic acid/water because of poor resolution of pipecolate, AMP, and pipecolate adenylate in this system. However, its formation was monitored by reaction with hydroxylamine and quantitated as the hydroxamate with ferric chloride reagent (Berg, 1958). Incubation with enzyme and $^{32}P[P]$ and analysis of radioactive products was performed as described previously (Keller et al., 1984).

Immunomycin Titers and Isolation Procedures. Analytical HPLC was done with use of a Whatman Partisil 5 ODS-3 analytical column equilibrated at 60 °C in a mobile phase of acetonitrile/0.1% H_3PO_4 (65:35) and run at 1.0 mL/min. Compounds of interest were detected at 205 nm. Retention times for immunomycin, FK 506, and rapamycin were 10.1, 10.6, and 11.4 min, respectively.

Washed-cell incubations were carried out with use of a modification of a published procedure (Nielsen & Kaplan, 1989). Cells from production medium were harvested at the

Table I: Purification of Pipecolate-Activating Enzyme[a]

	vol (mL)	protein (mg/mL)	specific activity (pmol/mg)	units (nmol)	% activity yield	fold purification
crude extract	274	12.8				
AS 30–65%	118	13.8	9.8	16	100	1
DE52	49	4.5	29.1	8.5	53	3
Seph-S300	52	1.3	63.6	4.2	27	7
total MQ pool	2.8	3.3	324	3.0	19	33
best MQ pool	0.7	2.2	530	0.8	5	54

[a] Assay is binding of [^{14}C]pipecolate as described in Materials and Methods.

beginning of product formation (24–30 h) and washed twice, and the cells were resuspended to the original volume in incubation buffer containing glucose at 1.0%, MOPS, pH 6.8, at 0.25%, and analogue at concentrations from 0.5 to 2 mg/mL. A volume of 30 mL of resuspended cells was placed in a nonbaffled shake flask (or 2.5 mL in a 25 × 150 mm tube) and shaken at 27 °C and 220 rpm for 18–24 h. The whole broth was extracted with an equal volume of methanol and centrifuged, and methanol was evaporated under reduced pressure. Extraction of the remaining aqueous mixture with an equal volume of ethyl acetate followed by drying over Na_2SO_4 and evaporation to dryness afforded the desired materials in crude form. After being washed with hexane, the resulting material was dissolved in methanol and purified by semipreparative HPLC on a Rainin Dynamax 10 mm × 25 cm column equilibrated at 50 °C in acetonitrile/0.1% H_3PO_4 (50:50), with a flow rate of 4.0 mL/min.

Results

Purification. Sonicated extracts of cells harvested at 60–68 h were examined for the ability to catalyze a pipecolic acid dependent PP–ATP-exchange reaction, after desalting by filtration on Sephadex G25 to remove amino acids. Before antibody was available, assays and enzyme purification were performed on extracts of this age, chosen on the basis of immunomycin accumulation. Cells harvested at 25–40 h would have been preferable, and in later enzyme preparations younger cells were used, as noted in the Discussion. DL-pipecolic and L-pipecolate both showed some stimulation of the fairly high endogenous PP–ATP-exchange rate in desalted cell-free extracts. The stimulation was a function of substrate concentration up to about 5 mM in the case of L-pipecolate and 10 mM for DL-pipecolate. This suggests that the response was due to the L form and was not inhibited by the presence of the D isomer. No convincing evidence of binding of pipecolate to protein in an acid-stable form could be found in crude extracts. However, we proceeded to purify the exchange activity and found that after a concentrative step with ammonium sulfate and elution from DEAE-cellulose we could resolve the endogenous exchange activity from a fraction exhibiting pipecolate-dependent PP–ATP-exchange activity. Stimulation by L-proline, at its optimal level, was about 5% of the stimulation by pipecolate. This enzyme fraction also had appreciable binding capacity for [^{14}C]pipecolate (Table I). The characteristics of this binding will be discussed below.

In further purification, binding and exchange activities cochromatographed in every procedure tried. After a shallow gradient on MonoQ, the most prominent proteins were a pair of bands seen in Figure 2, lane 4, migrating a little slower than the β and β' subunits of RNA polymerase 155 and 165 kDa. The degree of resolution of the two bands was dependent on both the amount of protein loaded and the pH of the electrode buffer. Prolonged electrophoresis actually resolved this apparent pair into a family of bands. N-Terminal sequencing

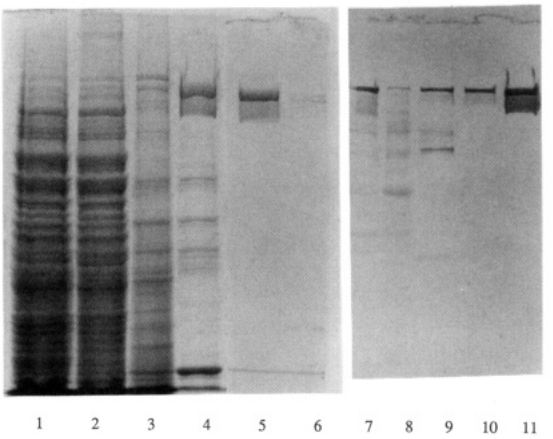

FIGURE 2: Purification of the pipecolate enzyme: lanes 1–6, Coomassie stained; lanes 7–11, Western blotted; lanes 1 and 7, crude extract; lanes 2 and 8, ammonium sulfate 30–65%; lanes 3 and 9, DEAE-cellulose pool; lanes 4 and 10, MonoQ pool; lanes 5 and 11, electrophoretically purified pool; lane 6, 1 μg of *E. coli* RNA polymerase showing bands at 165, 155, and 39 kDa. The gels used were 8% SDS/Tris/glycine gels from Novex.

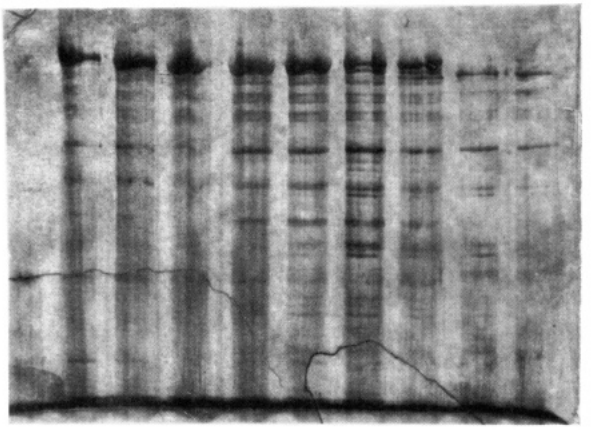

FIGURE 3: Fingerprinting of separate regions of the 170-kDa family of bands. The original gel electrophoresis was performed in a 6% gel run until the 170-kDa band was near the bottom. After brief staining in Coomassie blue, four thin regions were cut from the slowest to leading edge, A, B, C, and D, respectively, divided in half vertically, digested in the wells of a 12% SDS gel prepared in a Hoefer apparatus with V8 protease for 30 min as the dye front was entering the running gel as described in Cleveland et al. (1977). Final staining was with a Bio-Rad silver kit. Lane 1, 60 ng of V8 protease; lane 2, A plus 20 ng of V8; lane 3, A plus 60 ng of V8; lane 4, A without V8; lane 5, B plus 60 ng of V8; lane 6, B plus 20 ng of V8; lane 7, C plus 60 ng of V8; lane 8, C plus 20 ng of V8; lane 9, D plus 60 ng of V8; lane 10, D plus 20 ng of V8.

revealed multiplicity even in the upper apparently single band. Fingerprinting with V8 protease showed that all bands are closely related to each other (Figure 3). Heterogeneity was even more pronounced if serine protease inhibitors were not

Table II: Effect of NEM on the Binding of Pipecolate to the Purified Activating Enzyme

	picomoles of pipecolate bound	
	no pretreatment	pretreated with 2 mM NEM
standard binding assay[a]	2.3	0.02
sephadex G50 exclusion[b]		
total	4.1	2.1
pellet after TCA precipitation	2.5	0.04
TCA supernatant	1.7	2.0

[a] As described in Materials and Methods. [b] Rapid filtration by Sephadex G50 was performed in 5-mL columns in 25 mM HEPES/10% glycerol/0.2 mM DTT. TCA treatment was for 30 min at room temperature in the presence of additional carrier protein. NEM treatment was for 2 min prior to the addition of ATP, $MgSO_4$, DTT, and [^{3}H]pipecolate.

included in the extraction buffer or if 0.2 mM PMSF was not included in buffers for all steps prior to MonoQ chromatography. The addition of 1 mM 1,10-phenanthroline to extraction and purification buffers made no difference. We concluded that the band multiplicity was due to proteolytic "fraying", despite the inclusion of a collection of serine protease inhibitors in the early steps. The final heterogeneity in enzyme from cells harvested at 40 h, or even earlier at 25 h, was not significantly less than in the earlier preparations performed with cells harvested at 60–68 h. Association of activity with the whole 170-kDa cluster of proteins was demonstrated by nondenaturing gel electrophoresis followed by cutting of the lane into 2-mm slices, elution, measurement of restored exchange assay, and finally SDS gel electrophoresis (results not shown). This, however was not a useful preparative step because of the proximity of other bands under native conditions. Since many chromatographic procedures failed to purify the activity significantly more than the MonoQ fractions, we resorted to SDS electrophoretic purification for the last step. Both bands showed heterogeneity when separately blotted for N-terminal sequencing, despite the migration of the upper band as an apparently single species. Both bands were included in the region eluted because both are active in the exchange assay when fractionated from nondenaturing gels, and they share the same fingerprint pattern. Coomassie stain of the final preparation is shown in Figure 2, lane 5. This preparation was used to raise antibodies. Western blots of successive steps in the purification are shown in Figure 2, lanes 6–10. The increase in the lower diffuse band during isolation suggests that it is, at least in part, the result of cleavage after cell disruption. It migrates as a single diffuse band at pH 5.2 upon isoelectric focussing.

Characteristics of the Binding Reaction. Binding of pipecolic acid in a TCA-precipitable form requires ATP and Mg^{2+}, is very rapid, and is abolished by the sulfhydryl reagents *N*-ethylmaleimide (NEM) or dichlorodithionitrobenzoate. The TCA-precipitable product is stable to formic acid but unstable to performic acid, releasing the radioactivity in a species that comigrates with pipecolic acid on TLC (Figure 4). These are properties consistent with those of an enzyme-bound thioester, whose formation is preceded by noncovalent binding of an amino acid adenylate. Such thioesters have been described for a large number of amino acid activating enzymes in peptide and peptidolactone biosynthesis (Kleinkauf & von Dohren, 1987).

Pipecolate Adenylate as an Enzyme-Bound Intermediate. Pipecolate binding was also examined with use of rapid gel filtration on Sephadex G50 to separate enzyme-bound from

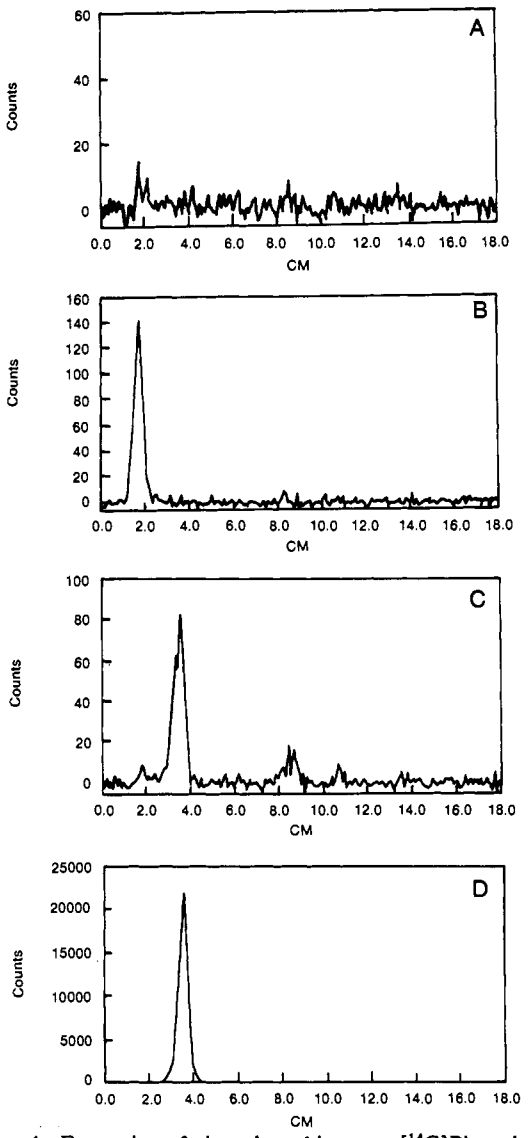

FIGURE 4: Formation of pipecolate thioester. [^{14}C]Pipecolate was incubated with MonoQ-purified enzyme as described in Materials and Methods. The washed pellet was dissolved in formic acid and examined by TLC on silica plates developed in butanol/acetic acid/water 60:15:15. The distribution of counts was determined by Bioscan. Panal A, incubation without ATP; panel B, complete incubation; panel C, complete incubation but pellet was pretreated with performic acid before dissolving in formic acid; panel D, [^{14}C]pipecolate standard.

free pipecolate. Both acid-labile and acid-stable counts were found at nearly equimolar levels in the excluded volume (Table II). Similar behavior has been described for gramicidin S synthetase 1, where the acid-labile counts are ascribed to an enzyme-bound adenylate (Kanda et al., 1981). The formation of acid-labile counts was insensitive to the sulfhydryl-blocking reagent NEM, in contrast to the acid-stable counts comprising the aminoacyl thioester (Table II).

Further evidence for pipecolate activation through the adenylate was obtained by incubating chemically synthesized pipecolate adenylate with purified enzyme and labeled pyrophosphate (Figure 5). Radioactive ATP was formed, as well as a small amount of ADP. The latter was reduced by adding cold pyrophosphate (Keller et al., 1984).

Size. Gel filtration of the pipecolate-activating enzyme purified to the MonoQ stage showed that the enzyme is a dimer of approximately 300 kDa. Small amounts of a protein of about 300 kDa, positive on Western blotting, can be seen in some Coomassie blue stained SDS gels of the purified en-

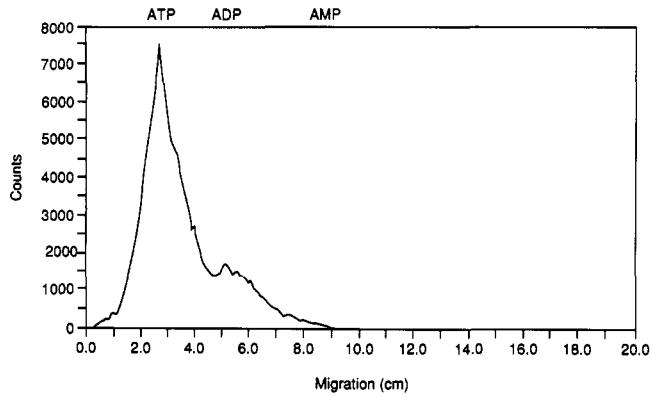

FIGURE 5: Reversal of adenylation reaction with pipecolate adenylate. A total of 30 pmol of enzyme was incubated for 20 min with 60 pmol of [^{32}P]P (5 × 10^5 dpm) and 15 nmol of adenylate followed by adsorption of nucleotide onto charcoal. A control with the same levels of enzyme, pyrophosphate, and pipecolate plus AMP showed no labeled nucleotide product.

Table III: The Ability of Proline and Pipecolate Analogues To Stimulate the PP–ATP-Exchange Reaction

	% of L-pipecolate activity[a]
L-proline	19
L-4-hydroxyproline	12
X-hydroxyproline (*X* = 1, 2, or 3)	12
trans-3-methyl-L-proline	19
cis-3-methylproline	5
cis-3-methyl-DL-proline	4
cis,trans-4-methylproline	178
cis-4-methyl-DL-proline	4
trans-4-aminoproline	6
cis-4-chloro-L-proline	33
5-iminoproline hydrochloride	16
cis-5-methyl-DL-proline	25
(+)-piperazic acid	24
5-chloropipecolate	32
5-hydroxypipecolate	7
cis-4-hydroxy-L-pipecolate	16
trans-4-hydroxy-D-pipecolate	6
4-hydroxyallopipecolate	19
thiazolidine-4-carboxylic acid (thioproline)	4

[a] 100% = initial rate of PP–ATP exchange with L-pipecolate at 10 mM (1.3 mg/mL), which is a saturating level. The analogues were all tested at 2.5 mg/mL.

zyme even after treatment with a large excess of mercaptoethanol or DTT. It disappears upon carboxymethylation prior to gel electrophoresis and thus appears to result from a tendency for the purified enzyme to dimerize even under reducing SDS conditions.

The isoelectric point of the purified enzyme, as determined on Phast isoelectric focusing gels, is about 5.2. At this pH the enzyme is quite unstable, exchange activity exhibiting a half-life of about 30 min at 4 °C.

Specificity. The naturally occurring amino acids and pipecolate and proline analogues were tested for their ability to catalyze the PP–ATP-exchange reaction exhibited by the purified pipecolate-activating enzyme (Table III). Of the amino acids found in proteins only L-proline is active. D-Proline is devoid of activity. Substitutions on the pipecolate ring reduce activity, while those in proline have variable effects. Trans substitution at the 4-position of proline by a methyl group enhances activity above that of pipecolate. L-Azetidine-2-carboxylic acid, which can replace proline in gramicidin S biosynthesis (Stoll et al., 1970), is completely inactive.

Activity in the exchange reaction is presumably necessary but not sufficient to ensure adenylation or thioester formation,

Table IV: Affinity Constants[a] Determined for Binding of [^{3}H]Pipecolate in the Standard Assay

	K_m(binding) (μM)	IC_{50}(pipecolate binding) (mM)
L-pipecolate	0.4	
L-proline		3.0
4-methylproline (cis,trans)		0.15
3-methylproline (trans)		1.2
5-hydroxypipecolate		1.1
thioproline		1.2

[a] The inhibition constants were obtained by adding increasing amounts of analogue to binding reactions containing 3.5 μM [^{3}H]pipecolate.

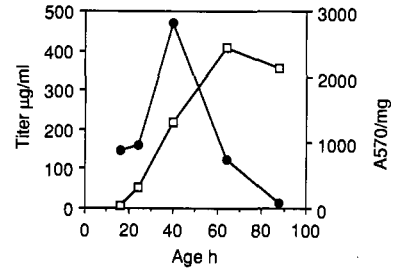

FIGURE 6: Correspondence of time course of antigenically detectable pipecolate-activating enzyme with immunomycin productivity. Crude extracts were prepared from equal volumes of culture at the indicated times and saved as heated extracts in Laemmli buffer for running and Western blotting in a single gel. The resulting alkaline phosphatase stained bands were scanned at 570 nm and area normalized for protein in each extract, given as filled circles. The open squares represent titer.

let alone subsequent transfer to the acceptor in immunomycin synthesis. Exchange rates with analogues are not necessarily correlated with the rate of adenylation (Keller et al., 1984) and reaction at the thiol site often shows greater specificity than either exchange or adenylation reactions (Katz & Demain, 1977). In vitro assay, first by exchange and then by competition in pipecolate binding, is useful as a rapid screen for those compounds most likely to yield immunomycin analogues in vivo. Several of the analogues most active in the exchange reaction act as inhibitors of pipecholate binding. Much higher concentrations than that of pipecolate are required, as can be seen from the inhibition constants in Table IV. In directed biosynthesis experiments using whole cells, the addition of high levels of proline causes the synthesis of some prolylimmunomycin (patent filed), indicating that proline can be inserted in vivo, despite the apparently very unfavorable binding constant. We do not detect the proline analogue of immunomycin in normal fermentations, although the prolyl derivative of FK 506 (FR-900525) has been reported by Fujisawa (Hatanaka et al., 1988b) as a minor component in fermentation broths. Surprisingly, the addition of high levels of thioproline also causes the accumulation of prolylimmunomycin. This result is not due to contamination of thioproline by proline, and at present we cannot explain it. Thioproline inhibits pipecolate activation but is no more potent than 3-methylproline or 5-hydroxypipecolate, which do not cause the accumulation of prolylimmunomycin. Fermentations of MA 6678 with 4-methylproline give rise to a new peak in the HPLC profile, but its identity has not yet been established.

Immunological Screening. Western blots of crude extracts of MA 6678 were performed at various harvest times up to 88 h, when immunomycin titers have reached a plateau. A densitometry scan of the 170-kDa complex, stained via a second antibody and alkaline phosphatase, is compared with product accumulation in Figure 6. The period of maximal

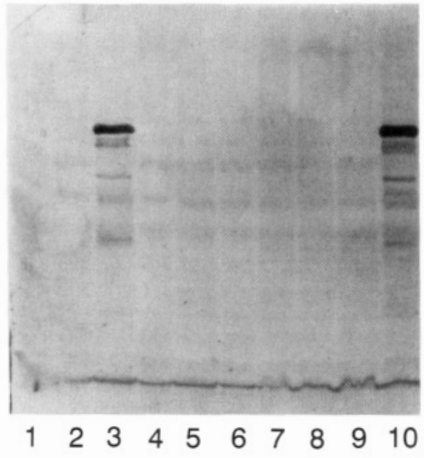

FIGURE 7: Western blot of nine immunomycin non-producers, lanes 1–9, and the immunomycin-producing strain MA 6678, lane 10. A total of 25 μL of crude extracts, containing 180–220 μg of protein, was electrophoresed on a 6% Laemmli SDS gel.

detectible immunoreactive bands, between 25 and 40 h, corresponds to the highest immunomycin synthetic rates. When the production rate decreases after 40 h, the intensity of immunoreactive protein declines rather than stays constant. Whether this reflects regulation in vivo or is an artifact of cell breakage cannot be determined at present.

The immunomycin producer (MA 6678) was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and 12 nonproducers were obtained following screening by thin-layer chromatography. The IL2 assay (Dumont et al., 1990) and HPLC profiles of fermentation broths confirmed the nonproductivity of these mutants. They were screened by Western blotting for the presence of the pipecolate-activating enzyme. Ten were completely negative, while two produced normal levels of the 170-kDa complex of proteins. A Western blot of crude extracts from the eight negative strains and one positive nonproducer are shown in Figure 7, along with the parental culture MA 6678.

Streptomyces strains producing the related compounds FK 506 and rapamycin, both of which contain a pipecolate residue, were examined for immunoreactive bands with use of the antibody raised to the pipecolate-activating enzyme from *S. hygroscopicus var. ascomyceticus*. Despite optimizing harvest times and rapid working to minimize proteolysis, no cross-reactive bands have been found in these cultures. The strains available for testing for cross-reacting proteins accumulate much less peptidolactone than the immunomycin producer we are using, which may well contribute to our failure to find immunoreactive bands.

Discussion

This work was prompted by our ongoing effort to study the biosynthesis of the FK 506 family of immunosuppressants. Lysine was shown to be the precursor of pipecolate in the related compound rapamycin (Paiva, 1988). The results of similar isotope experiments in the immunomycin producer suggested the incorporation of an intact unit of pipecolate derived from lysine catabolism into immunomycin. DL-[U-^{14}C]Lysine was incorporated by washed cells into immunomycin in a manner readily diluted by low levels of pipecolate. Further experiments with DL-[1-^{13}C]lysine established the specificity of this incorporation. An 80-fold enrichment at C1 of immunomycin was found, with very little enrichment elsewhere, as expected if lysine is the precursor of pipecolate (Byrne et al., 1991). The ε-amino group of lysine is retained in the cyclization reaction. Retention of the ε-amino group seems general in bacterial systems for pipecolate synthesis (Fothergill & Guest, 1977), while in some fungi an alternate route retaining the α-amino group is used (Wickwire et al., 1990).

The attachment of pipecolate to immunomycin is through an amide bond with the carboxyl end of an acetate unit. This mode of attachment suggests that an amino acid activating enzyme may be involved, and this proved to be a fruitful approach to otherwise difficult enzymology. Indeed an enzyme with properties very similar to those now well characterized for nonribosomal peptide antibiotic synthesis was found in cell-free extracts of the immunomycin producer *S. hygroscopicus var. ascomyceticus*. Assays developed for activating enzymes involved in peptide or peptidolactone biosynthesis in other systems were used to isolate an enzyme activating pipecolic acid and to a lesser extent proline and some pipecolate analogues. Several chromatographic purification steps and a final step of preparative electrophoresis yielded a preparation with multiple N termini migrating in SDS gels as a broad band at about 170 kDa and a diffuse band a little faster. All the bands share a common fingerprint pattern and are presumably derived from a single sequence. The faster bands appear to be derived from the slower moving bands, since their amount increases during purification. Antibody to the purified pipecolate-activating enzyme was used to examine enzyme concentration during tropophase and idiophase by Western blotting. The pipecolate-activating enzyme is most abundant at the time of maximal synthesis of immunomycin at the beginning of idiophase. Moreover, it is absent from a number of nonproducing mutants, providing additional evidence for its role in immunomycin biosynthesis. The high frequency (10/12) of strains lacking immunoreactive protein among the independently derived nonproducers tested was surprising. The reason for such a frequency may become clear upon genetic analysis.

The properties of the purified pipecolate-activating enzyme are typical of amino acid activating enzymes already characterized for the synthesis of purely peptide products. We found evidence of an adenylation reaction, exhibiting a vigorous PP–ATP exchange dependent upon L-pipecolic acid. An acid-labile pipecolate-containing intermediate as well as an acid-stable pipecolate-containing species with the properties of a thioester was demonstrated. Furthermore, reversal of the adenylation, using chemically prepared pipecolate adenylate to produce ATP from pyrophosphate, added additional evidence of pipecolate adenylate as an intermediate in the activation reaction. The specificity of the activating enzyme is consistent with a role in activating primarily L-pipecolate and to a much lesser degree L-proline. Feeding proline to washed cells allowed the detection of the proline analogue of immunomycin. Characterizing the specificity of the pipecolate-activating enzyme has permitted some predictions of which analogues of pipecolate might be incorporated, but clearly pools of pipecolate and proline prevailing during fermentation are also determining factors of whether an analogue is appreciably incorporated in vivo.

The size of the pipecolate-activating enzyme, about 170 kDa as monomer and aggregated apparently as a dimer under native conditions, is larger than usual for an enzyme activating a single amino acid. A recent survey of amino acid activating enzymes for nonribosomal peptide synthesis (Kleinkauf & von Dohren, 1990) lists 120 kDa as the largest subunit activating one amino acid. This enzyme, the GS1 of gramicidin S synthesis, effects racemization of phenylalanine in addition to its activation. Monofunctional activating enzymes are more

usually 45–60 kDa. It is possible that the pipecolate-activating enzyme has additional functions in the biosynthesis of immunomycin; this awaits further investigation.

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