Penicillin Biosynthesis: Energy Requirement for Tripeptide Precursor Formation by δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine Synthetase from *Acremonium chrysogenum*†

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ABSTRACT: In nonribosomal peptide formation by multifunctional enzymes, peptide synthetases catalyze the activation and directed condensation of amino acids. The peptide synthetase involved in penicillin biosynthesis (ACV synthetase) forms the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine from the respective L-amino acids and ATP. So far, the energy requirements for the nonribosomal process have not been clearly established. For ACV synthetase we show that ATP consumption depends on the reaction conditions employed. By simultaneously estimating peptide and AMP production by employing fluorescence detection and UV spectroscopy, respectively, we have determined the energy consumption with high accuracy. Under unfavorable reaction conditions more than 20 mol of ATP are consumed/mol of tripeptide formed, while optimal conditions permit the expected energy requirement of one ATP for each carboxyl group activation, corresponding to three ATP for tripeptide formation. The third ATP is required for the activation of L-valine to maintain the valyl-thioester stage for epimerization and peptide bond formation, and this high-energy bond is sacrificed by hydrolytic removal of the product. No extra energy is required for the directed transport in peptide elongation. Additional energy consumed has been traced to hydrolytic loss of activated intermediates, as has been shown by the analysis of incomplete reaction mixtures.

Peptide biosynthesis proceeds either ribosomally or by nonribosomal enzymatic pathways. The nonribosomal pathways can be grouped into amino acid adding systems and multistep polyenzyme systems (*1*, *2*). While amino acid adding systems activate either amino acid or peptide carboxyl groups and catalyze the addition of the amino group of an amino acid, peptide synthetases generally catalyze the activation and directed condensation of amino acids.

In the ribosomal system ATP[1] is required for the synthesis of aminoacyl-tRNA catalyzed by the respective aminoacyl-tRNA synthetases. In addition, proofreading in the aminoacylation process of tRNA charging may consume several additional molecules of ATP, depending on the respective amino acylation system (*3*). Further energy is required for each elongation step for translocation of the involved macromolecules including mRNA (*4*). One GTP is bound to elongation factor TU (EF-TU) in a ternary complex with aminoacyl-tRNA which enters the acceptor site of the ribosome and is hydrolyzed before peptide bond formation occurs. A second GTP is required for the translocation of peptidyl-tRNA from the ribosomal A (aminoacyl) to the P (peptidyl) site. By changing the substrate specificity of EF-TU to xantosine triphosphate (XTP) by employing site-directed mutagenesis, Weijland and Parmeggiani (*5*) have shown the consumption of 2 XTPs per synthesized peptide bond.

In the nonribosomal process Fujikawa et al. have shown in their pioneering work (*6*) an approximate requirement of one ATP per peptide bond using a crude system forming the cyclodecapeptide tyrocidine. Biosynthesis was determined by radiotracer incorporation, subtracting as a control the ATPase activity in the absence of amino acids. The reaction mixtures thus contained very low concentrations of amino acids (50 nmol in a total volume of 500 μL) as well as ATP (1 μmol), and incubations were performed for more than 30 min without ensuring rate linearity.

However, other studies using bacitracin and mycobacillin biosynthesis determined a consumption of 2 mol of ATP per peptide bond (*7*, *8*). Peptide formation was analyzed by radiochemical assays limiting the concentration of at least one of the amino acids. In both cases, ATP concentration dependence was measured. Subsequent work on the gramicidin S system has shown a less defined energy requirement, estimating an operational ATP consumption between 1 and 2 mol per peptide bond formed (*9*).

We have chosen as a model system the peptide synthetase involved in penicillin biosynthesis (*10*), δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS). This multienzyme has been shown to catalyze tripeptide formation from the

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[1] Abbreviations: A and Aad, α-aminoadipic acid; ACV, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine; ATP, adenosine triphosphate; AMP, adenosine monophosphate; C, cysteine; DTE, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; S, L-serine; V, L-valine; D-V, D-valine, HPLC, high-pressure liquid chromatography; P, product(s).

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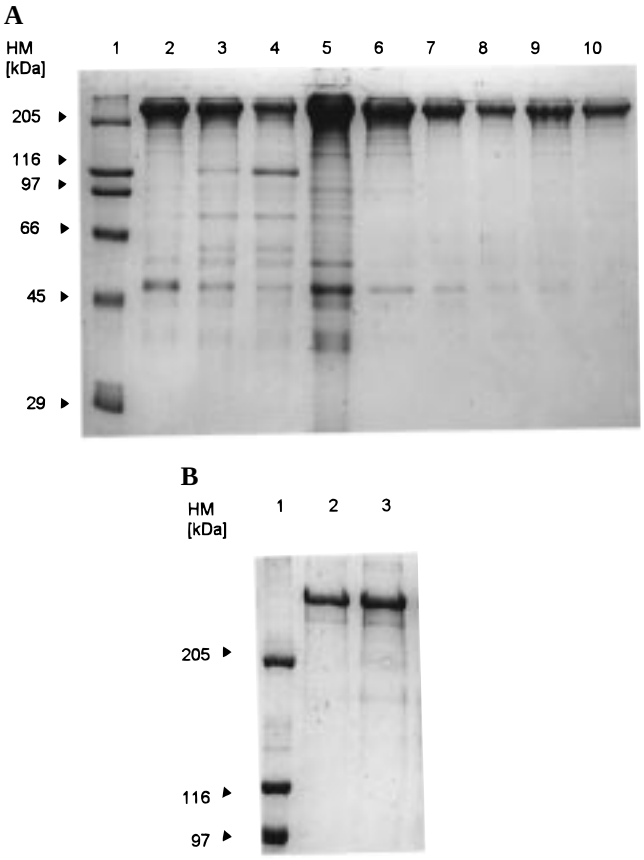


FIGURE 1: (a) Purification of ACV synthetase from *Acremonium chrysogenum*. SDS−PAGE (10%) analysis. Lanes: 1, Highmarker; 2−4, AcA 34 fractions; 5, ACVS after concentration with Amicon cell (1 M NaCl); 6−10, Superdex 200 gel filtration fractions. (b) Purification of ACV synthetase from *Acremonium chrysogenum*. SDS−PAGE (5%) analysis. Lanes: 1, Highmarker; 2−3, AcA 34 fractions.

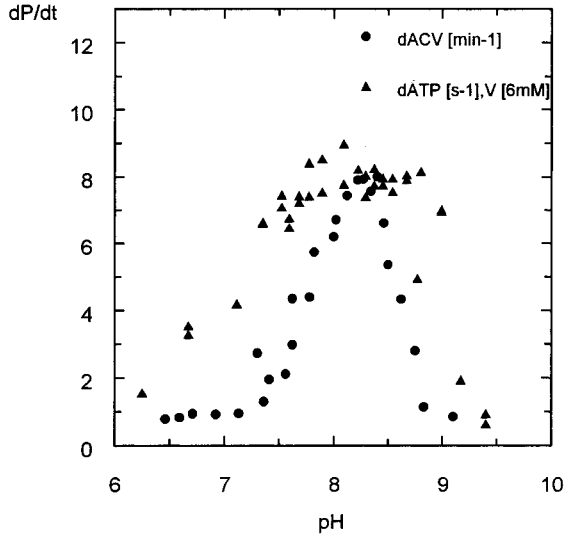


FIGURE 2: pH dependence of ACV biosynthesis compared to valine-dependent ATP/PPi exchange. Reaction conditions for ACV biosynthesis: A (1 mM), C [1 mM], V [3 mM], ATP [8 mM], Mg^{2+} [40 mM], ACVS [0.39 μM], bicine [70 mM], and DTE [6.6 mM], 1 h, 29 °C. Adenylation of L-valine: V [6 mM], ATP [0.1 mM], Mg^{2+} [24 mM], PPi [0.05 mM], [^{32}P]PPi [0.1 μCi], ACVS [0,2 μM], DTE [2 mM], 30 min, 29 °C. P, products (ATP, ACV).

respective L-amino acids and ATP. ACVS contains three amino acid binding sites and at least three ATP binding sites. To approach a kinetic description of the system, we have attempted to first establish the stoichiometry of the overall reaction.

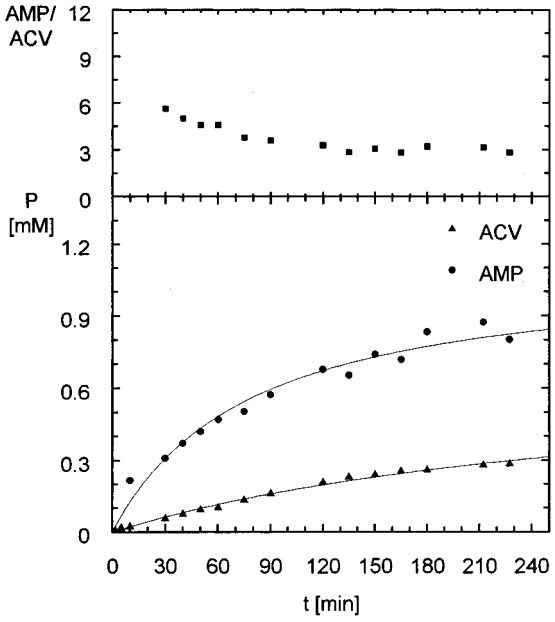


FIGURE 3: Full time curve of ACV biosynthesis. Reaction conditions: A [1 mM], C [1 mM], V [3 mM], ATP [8 mM], Mg^{2+} [40 mM], ACVS [0.39 μM], bicine [70 mM], pH 8.4, and DTE [6.6 mM], 29 °C. P, products (AMP, ACV).

According to the currently accepted model of the thiotemplate mechanism of peptide biosynthesis by multienzymes, one α,β-phosphate bond of ATP is consumed for the formation of one peptide bond (*11*). However, it has not been shown whether the directed transport of intermediates requires additional energy.

In this work we have analyzed ATP consumption using a fluorescence detection system for the peptide coupled to AMP and ATP determination.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions. Enzyme was prepared from *Acremonium chrysogenum* C-10. Cells were grown as previously described for *Aspergillus nidulans* (*10*). Fermentations were seeded with an overnight culture instead of a spore suspension. The mycelia were harvested 40 h after inoculation, before the period of transition to the stationary growth phase. The mycelia were washed with 0.8% NaCl solution, lyophilized, and stored at −20 °C under dry conditions.

Enzyme Purification. All procedures were carried out at 0−4 °C in succession. Fifty grams of dried mycelia was ground in a mortar with sand and stirred for 40 min in 1 L of buffer A (100 mM Tris/HCl, pH 7.5, at 4 °C, 10 mM DTE (Biomol), 1 mM EDTA, and 50% glycerin). After 30 min of centrifugation at 10000*g*, nucleic acids were precipitated from the supernatant for 20 min with dialyzed Polyimin solution (BASF, Ludwigshafen), final concentration 0.1%. This solution was centrifuged as above, and to the supernatant was gradually added neutralized saturated ammonium sulfate solution to a final concentration of 30% saturation. The solution was stirred for 20 min and centrifuged. Subsequently, ammonium sulfate solution was added to the supernatant up to a concentration of 45%, and the pellet after centrifugation was carefully dissolved in buffer B (25 mM

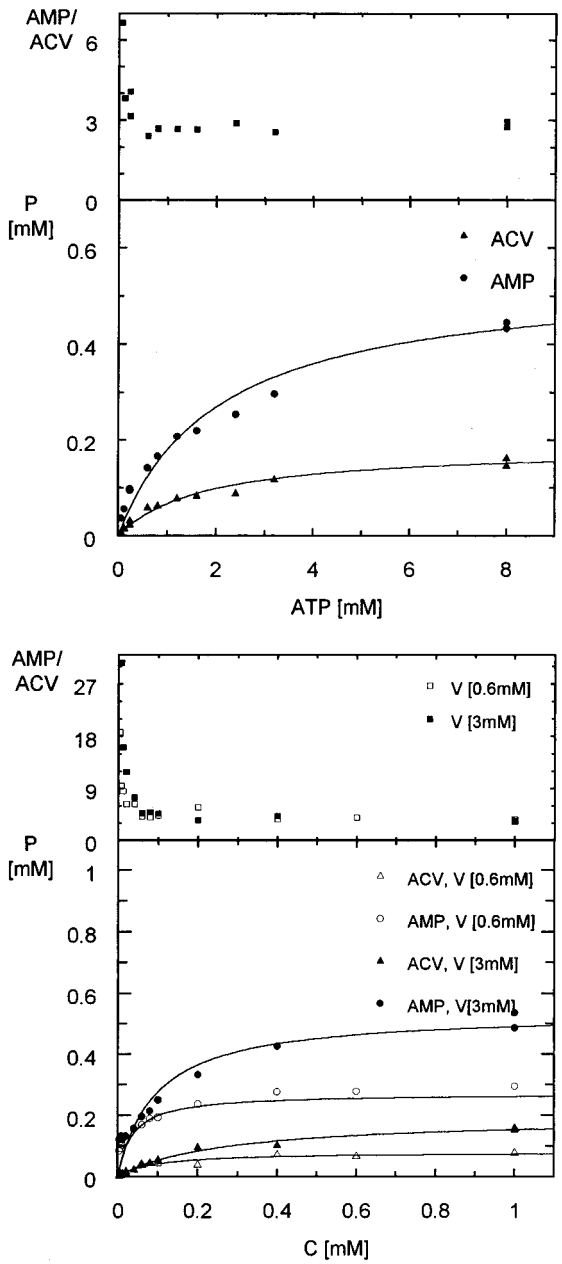


FIGURE 4: (a) Energy requirement depending on various concentration of ATP. Reaction conditions: A [1 mM], C [1 mM], V [3 mM], Mg^{2+} [40 mM], ACVS [0.39 μM], bicine [70 mM], and DTE [6.6 mM], pH 8.4, 1 h, 29 °C. P, products (AMP, ACV). (b) Energy requirement depending on concentration of cysteine. Reaction conditions: A [1 mM], V [3 mM], ATP [8 mM], Mg^{2+} [40 mM], ACVS [0.39 μM], bicine [70 mM], and DTE [6.6 mM], pH 8.4 1 h, 29 °C. P, products (AMP, ACV).

Tris/HCl, pH 7.5, at 4 °C, 0.1 EDTA, 2 mM DTE). If the suspension was turbid, it was dialyzed for 1.5 h and centrifuged. Afterward the solution (7 mL) was applied to an AcA 34 (Serva) gel filtration column (5 × 50 cm). The ACVS-containing fractions were analyzed by SDS−polyacrylamide gel electrophoresis for protein composition. As shown in Figure 1the enzyme was about 90% pure after the first gel filtration step. Taking into account the high molecular weight of ACV synthetase (425 kDa), purification was continued with a second gel filtration step on a Superdex 200 (Pharmacia; Figure 1A, lanes 6−10) following a concentration step using a 50-mL Amicon cell (Figure 1, lane 5). Western blot analysis with the peptide synthetase

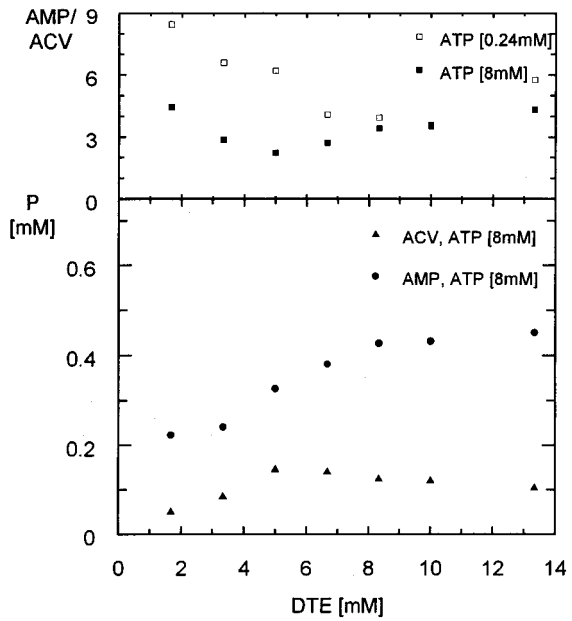


FIGURE 5: Energy requirement and product formation depending on the concentration of DTE. Reaction conditions: A [1 mM], C [1 mM], V [3 mM], Mg^{2+} [40 mM], ACVS [0.39 μM], and bicine [70 mM], pH 8.4, 1 h, 29 °C. P, products (AMP, ACV).

specific antibody [SGTTGKPKG (*12*)] showed a reaction only with the 425-kDa band of the fractions from both gel filtrations. SDS−PAGE gel (5%) was used to demonstrate AcA 34 fractions consisting of only a single enzyme with a molecular weight over 400 kDa (see Figure 1B).

The specific activities corresponding to the 425-kDa protein were checked by ATP/PPi exchange for the responsible amino acids (A, C, and V; data not shown). On the basis of these data, it was decided to use for kinetic studies the fractions from the AcA 34 gel filtration containing 90% pure ACV synthetase, which were frozen in liquid nitrogen in 1-mL portions and stored at −20 °C. The final enzyme concentrations used for kinetic studies were defined by comparing different methods [(*13*); extinction coefficient at 280 nm from ACVS (*14*)].

Standard Enzyme Assay. A reaction mixture containing 1 mM L-α-aminoadipic acid, 1 mM L-cysteine, 3 mM L-valine, 8 mM ATP, 40 mM Mg acetate (Sigma), 6 mM DTE, 70 mM bicine (Merck), pH 8.4, and approximately 0.5 μM ACVS was incubated for 1 h at 29 °C. The reaction was stopped with 100% TCA up to the final concentration of 5%, and the mixture was centrifuged for 5 min at 12000*g*. One aliquot was taken for the ACV derivatization, and another was neutralized with 200 mM MOPS for the ATP assay.

HPLC Assay. The assay is a modified procedure from Jensen et al. (*15*). First, ACV was derivatized with monobromobiman (Thiolyte reagent, Calbiochem). To a 25-μL sample was added 75 μL of derivatization mix [0.2 M EPPS/HCl, pH 8.0, 5 mM diethylentriaminpentaacetic acid (DPTA), and 3 mM monobromobiman]. The sample was incubated for 15 min in the dark, and the reaction was stopped with 12.5 μL of 1 M methanesulfonic acid. A 90-μL aliquot was diluted with HPLC buffer A (0.025% acetic acid/NaOH, pH 5.0). These samples were stable for 24 h at room temperature, so they could be analyzed with an autosampler.

For the analysis of ACV we used a reverse-phase C18 column (Waters, USA) eluting with a linear gradient from

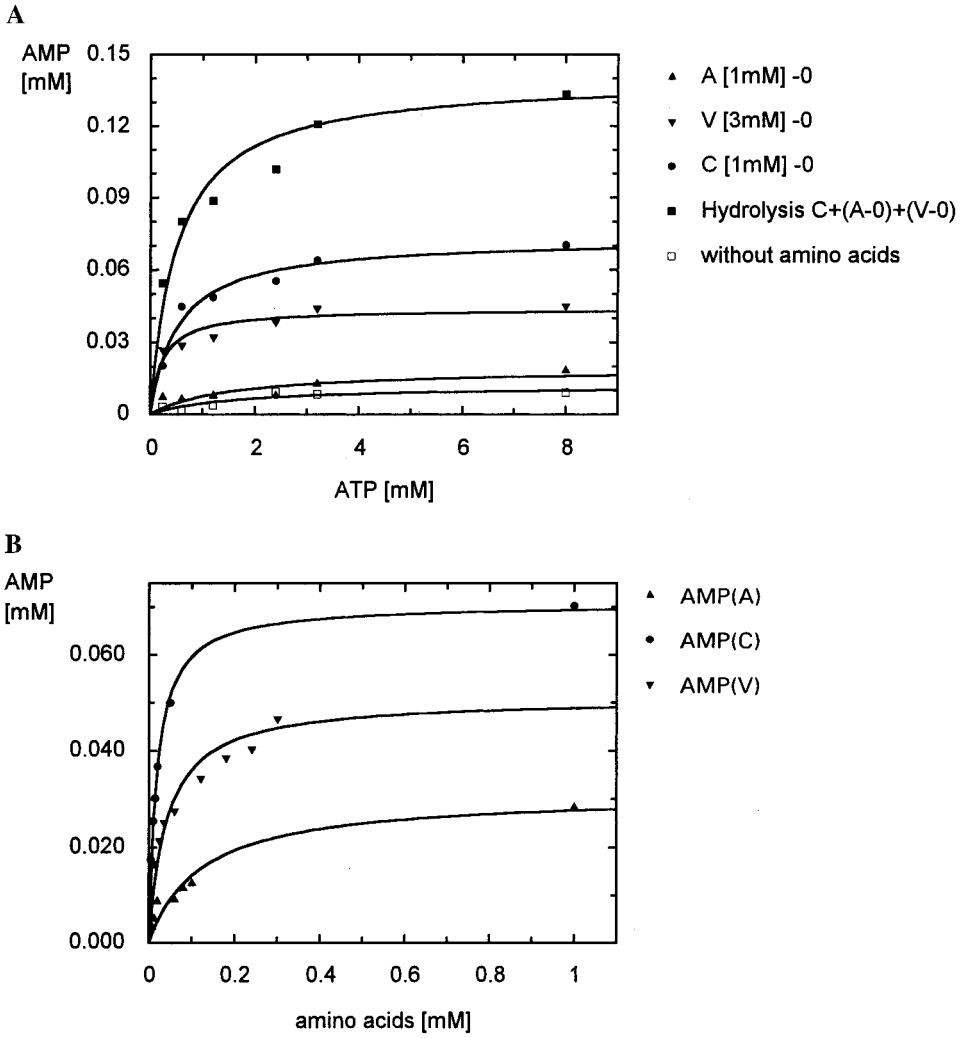


FIGURE 6: (a) Nonproductive hydrolysis of ATP in dependence on ATP concentration. Reaction conditions: Mg^{2+} [40 mM], ACVS [0.39 μM], bicine [70 mM], and DTE [6.6 mM], pH 8.4, 1 h, 29 °C. (b) Nonproductive hydrolysis of ATP dependent on amino acid concentration. Reaction conditions: ATP [8 mM], Mg^{2+} [40 mM], ACVS [0.39 μM], bicine [70 mM], and DTE [6.6 mM], pH 8.4, 1 h, 29 °C.

95% A (0.025% acetic acid/NaOH, pH 5.0) and 5% B (30% buffer A, 70% acetonitril) to 83% A and 17% B in 15 min and up to 100% B in another 10 min with a flow rate of 1.0 mL/min; registration of the fluorescence detection employed a 360-nm excitation filter and a 455-nm emission filter.

ATP and AMP were analyzed on a C18 column (Molnar, FRG) by isocratic gradient elution for 11 min with 100% A (10 mM KH_2PO_4, pH 6.0), followed by a linear gradient from 0 to 25% B (20% buffer A, 80% methanol) for 7 min, followed by 100% B with a flow rate of 0.7 mL/min; UV absorption was detected at 254 nm. The method was based on the procedure of Stocchi (*16*).

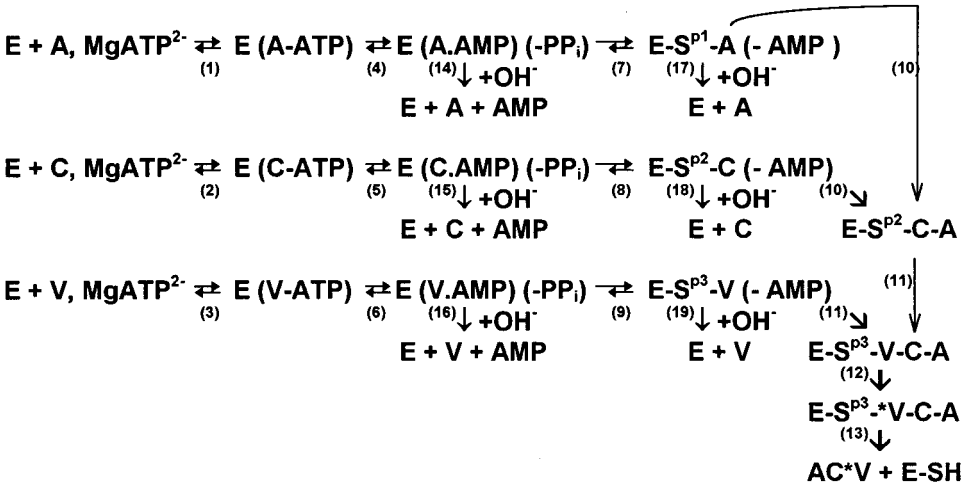
RESULTS

System Description. ACV synthetase has been isolated from freeze−dried *Acremonium chrysogenum* C-10 by a newly developed efficient two-step procedure. The material obtained by gel filtration is about 90% pure as judged by SDS−PAGE (Figure 1). Reaction conditions were analyzed for protein concentration dependence, which was linear in the range from 1 to 100 nM (results not shown). K_M values have been estimated for A (0.12 mM), C (0.09 mM), V (0.32 mM), and ATP (1.8 mM). A narrow pH optimum for ACV formation between pH 8.2 and 8.4 has been found, compared to a less pronounced pH dependence of the adenylation and thioester formation (optimum between pH 7 and 9) (Figure 2). A linear rate of ACV formation was observed for at least 120 min (Figure 3).

Time Dependence of Energy Requirement. To assess the stoichiometry of ATP consumption and ACV biosynthesis, we followed the kinetics of product formation (ACV, AMP). During 4 h of incubation a stable stoichiometry of 3:1 was demonstrated under saturating substrate concentrations (Figure 3). The higher ATP/ACV ratios in the initial phase are due to errors in the estimation at very low concentrations of ACV and AMP, respectively.

Dependence of Energy Requirement on ATP Concentration. ATP consumption was measured at saturating concentrations of the amino acid substrates. Formation of AMP and biosynthesis of ACV depend on ATP concentration. At 0.06 mM ATP a consumption of 6 mol of ATP/mol of ACV was measured, while at 8 mM ATP efficiency had improved to 3 mol/mol of ACV (Figure 4A). To estimate a possible loss of intermediates by hydrolysis of acyladenylates and thioesters, assays were performed with single amino acids, and AMP formation was determined (see Rates of Hydrolysis of Intermediates, below). Apparent Michaelis−Menten constants of 2 mM for ATP in relation to both products,

Scheme 1: Reaction Sequence Catalyzed by ACV Synthetase



1-3 enzyme substrate complex
4-6 aminoacyl adenylate complex
7-9 enzyme amino acid thioester
10-11 condensation (elongation)
12 epimerization of valine
13 hydrolysis of tripeptide
14-19 hydrolysis of intermediates

AMP and ACV, were equal within experimental error, connecting ATP hydrolysis and ACV biosynthesis. As expected, the catalytic constant for AMP (25 min^{-1}) was three times higher than for ACV (8 min^{-1}).

Dependence on Amino Acid Concentration. Probing the amino acid concentration upon limiting one amino acid in the reaction mixture, we obtained the same effect: at low concentrations the efficiency of energy requirement decreased, whereas at saturated conditions of all substrates the consumption is 3 ATPs per ACV tripeptide. The results for cysteine are shown in Figure 4B. K_M values for cysteine have the same range (0.1 mM) for ACV and AMP, while the catalytic constants are 8 and 25 min^{-1}, respectively. A higher consumption of ATP was observed, independent of the limiting amino acid and taking into account the ATPase activity of the enzyme from the control experiment (see below).

Effect of Thiol Reducing Agent (DTE). The possibility that the reducing agent DTE, which is required for the in vitro stability of ACVS, could promote the hydrolysis of intermediate thioesters was analyzed at two different ATP concentrations: at saturated conditions of 8 mM and in the linear dependence range of 0.24 mM (Figure 5).

The optimal concentrations of DTE between 5 and 8 mM for ACV biosynthesis correspond to an optimal energy requirement. The influence of ATP concentration confirms the decreasing efficiency under limited conditions.

Rates of Hydrolysis of Intermediates. The hydrolysis of intermediates was estimated by measuring the ATP consumption in incomplete reaction mixtures. Total ATPase activity of the enzyme was 0.7 min^{-1} in the absence of amino acids. This would correspond to 3% of the catalytic activity during ACV biosynthesis (25 min^{-1}); however, it is not known whether ATPase activity is actually relevant in the presence of substrate amino acids. Adding to the mixture one of the amino acids, we estimated remarkably higher ATPase activities: A, 0.8 min^{-1}; C, 2.6 min^{-1}; and V, 1.8 min^{-1} (Figure 6). Control measurements with two amino acids showed these catalytic constants to be nonadditive (turnover constants). Thus, independent of the combination of amino acids (Aad and Cys, Aad and Val, Cys and Val), maximal rates of hydrolysis were 3 min^{-1}.

Protein Stability. The possible degradation of ACVS has been investigated by SDS−PAGE (results not shown). No evidence for proteolysis has been found under reaction conditions.

Other Reaction Products. Reversal of adenylate formation by ATP could lead to P^1,P^4-di(adenosine-5′)tetraphosphate. No evidence for this compound has been found by HPLC analysis.

DISCUSSION

To estimate the stoichiometry between peptide formation and ATP consumption, we followed the concentrations of two products, ACV tripeptide and AMP. Optimal conditions permit the expected energy requirement of approximately 1 mol of ATP for each activated amino acid. In contrast to the ribosomal protein biosynthesis system, no additional energy is required for the directed transport in peptide elongation. The data, however, do not permit the estimation of the direct contribution of each possible side reaction to the observed excess energy consumption under suboptimal conditions. Limiting substrate concentrations lead to an increase of ATP consumption. This increase has been attributed to the hydrolysis of intermediates, as indicated in the reaction Scheme 1: reactions 14−16 (hydrolysis of adenylates) and 17−19 (hydrolysis of amino acid thioesters). Hydrolysis rates of up to 3 min^{-1} were determined in incomplete reaction mixtures. Hydrolysis might thus account for AMP generation under conditions of substrate limitations. As shown in Figure 4B at low concentrations of cysteine (below 0.05 mM) rates of ACV formation are below 0.5 min^{-1}, compared to 8 min^{-1} under saturating conditions. At

the same time AMP is produced at a rate of 5 min^{-1}. This unfavorable ratio of ATP consumption is even increased at high valine concentrations. It has been shown recently that the first and third domains of ACV synthetase expressed as fragments do not discriminate valine and aminoadipate (*17*, *12*). These results imply possible misactivations as a source of additonal energy consumption and suggest that rates of hydrolysis of misactivated sites may be elevated compared to preferred substrates, since products such as valylcysteinylvaline or aminoadipylcysteinyl aminoadipate are not observed, and misacylation is apparently prevented by corrective hydrolysis. Such a control could depend on the conformational change induced upon adenylate formation of the adjacent domain, and likewise the correction of misacylation could depend on missing conformational states promoted by domain interactions in peptide bond formation. Such a mechanism could explain the observed raise of AMP formation at high concentrations. In addition, abortive reactions could be added to the above scheme. So Shiau et al. (*18*, *19*) have isolated the dipeptide shunt products L-cysteinyl-D-valine and *O*-methylserinyl-D,L-valine in the presence of glutamic acid instead of L-Aad, or *O*-methylserine instead of cysteine. Rates of formation have been estimated at only 1 or 2% of peptide synthesis. However, all three substrates, which have been shown to form adenylates, were required for dipeptide formation. This has been interpreted as a conformational change of domain A by, for example, adenylate formation as being required for the reaction to proceed. Thus conformational changes in the presence of the added three substrates may alter velocities of hydrolysis with respect to rates observed in the system with one or two domains free of intermediates. Respective open and closed conformations have been proposed due to X-ray analysis of firefly luciferase, a member of the adenylate forming family, including luciferases, acyl-CoA synthetases, and peptide synthetases (*20*, *21*). The recent observation of the retainment of an ^{18}O-label in L-valine in the shunt product L,L-*O*-(methylserinyl)valine demonstrates the direct peptide bond formation of an aminoacyl adenylate. Thus amino groups might as well act as acceptors in side reactions of adenylates or thioesters. A frequent observation has been the modification of protein lysyl side chains by adenylate forming enzymes such as aminoacyl-tRNA synthetases and peptide synthetases such as tyrocidine synthetase (H. von Döhren and coworkers, unpublished results).

In the presence of the high concentrations of thiol reagents usually employed (e.g., 8 mM dithiotreitol), energy consumption significantly increases, probably due to the thiolysis of intermediates.

Biosynthesis of ACV presents a special case, since the formation of a tripeptide containing two peptide bonds requires 3 mol of ATP. Activation indeed is a requirement of the general thiotemplate mechanism, which is thought to proceed from two carrier-attached acyl intermediates at a respective adjacent condensation domain. The product formed remains carrier attached and could thus enter additional modification (like epimerization) or condensation reactions. In the case of ACV synthesis, the tripeptide is released after epimerization stereospecifically as LLD-ACV by the action of the C-terminal thioesterase (*22*). This mechanism implies another road to side products in analogy to the ribosomal system. In the nonribosomal system the peptide sequence is determined by the order of domains at the multienzyme level (*11*). At the respective condensation domains intermediate binding sites corresponding to the ribosomal A- and P-site have been proposed (*23*). Several observations on isolated multienzymes suggest intermediate starts by aminoacyl intermediates entering a presumptive P-site. This mechanism could account for this system (*18*, *19*) and for systems forming actinomycin (U. Keller, personal communication) and cyclosporin (R. Zocher, personal communication). Such reactions resemble initiation of the standard polyU-dependent poly-Phe translational system, with phenylalanyl-tRNA entering the P-site for initiation. The respective A- and P-site model is useful in the nonribosomal system to explain the direction of peptide bond formation.

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