Gramicidin S Synthetase. Temperature Dependence and Thermodynamic Parameters of Substrate Amino Acid Activation Reactions†

Joachim Vater,* Norbert Mallow, Sabine Gerhardt, Andrê Gadow, and Horst Kleinkauf
Institut für Biochemie und Molekulare Biologie, Technische Universität Berlin, D-1000 Berlin 10, West Germany
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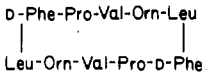
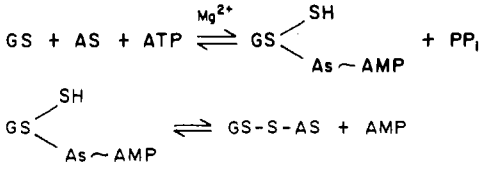
ABSTRACT: In the biosynthesis of the cyclic decapeptide antibiotic gramicidin S, the constituent amino acids are activated by a two-step mechanism involving aminoacyl adenylate and thio ester formation which are both reversible processes. The dissociation constants (K_D) for the gramicidin S synthetase–substrate amino acid–thio ester complexes are 100–1000-fold lower compared to the K_M data of the preceding aminoacyl adenylate reactions. The affinity for these substrates is appreciably higher at the thio template sites than at the aminoacyl adenylate reaction centers. Therefore, the activation equilibria are quantitatively shifted toward thio ester formation. A set of thermodynamic parameters for the activation processes was determined from the temperature dependence of the K_M and K_D data. Reaction enthalpies were obtained from a van't Hoff analysis of these constants. $\Delta G°$ for the substrate activation reactions of the heavy enzyme of gramicidin S synthetase (GS 2) is predominantly controlled by entropy contributions. In contrast, the overall activation and concomitant racemization of phenylalanine by phenylalanine racemase (GS 1) are exothermic processes which are distinguished by a small negative reaction entropy.

Various multienzymes that catalyze the biosynthesis of certain classes of peptide antibiotics, like the gramicidins, tyrocidines, bacitracins, etc., activate their substrate amino acids in a two-step mechanism (Lipmann, 1973; Laland & Zimmer, 1973; Katz & Demain, 1977; Kleinkauf & Koischwitz, 1978; Kleinkauf, 1981; Kurahashi, 1981). One of the best characterized systems among those multifunctional enzymes is gramicidin S synthetase. Gramicidin S is a cyclic decapeptide antibiotic of *Bacillus brevis* with the structure:



In the biosynthesis of this compound, two multifunctional enzymes cooperate, phenylalanine racemase [gramicidin S synthetase 1 (GS 1),[1] EC 5.1.1.11] and a condensing, phosphopantetheine-containing multienzyme (gramicidin S synthetase 2, GS 2). The first activation step for the constituent amino acids involves formation of aminoacyl adenylates. The preactivated substrates are then transferred to reactive SH groups at specific thio template sites which form the characteristic reaction elements to these multienzymes. Both activation steps represent reversible processes.



At present, knowledge is still lacking (a) regarding the specificity of substrate binding in particular at the thio templates and (b) regarding the factors in general that control the recognition of substrate amino acids at the reaction centers. Since adenylation and thiolation reactions are frequent elements in various enzymatic mechanisms, such information may be in addition of general significance. Aminoacyl adenylation, for example, is used for substrate activation both in peptide and in protein biosynthesis. Therefore, a comparison of the accuracy of substrate recognition of peptide synthetases on the one hand and tRNA synthetases on the other is of importance. The clue to an understanding of such factors is a detailed investigation of both thermodynamics and kinetics of substrate binding and product formation of these enzymes.

In this publication, we investigated the thermodynamic characteristics of amino acid activation by GS with the help of specific ATP–PP_i exchange experiments and thio ester binding studies.

Experimental Procedures

Materials. ^{14}C-Labeled amino acids and tetrasodium [^{32}P]pyrophosphate were purchased from Amersham/Buchler (Braunschweig); L-[^{3}H]ornithine was from NEN (Dreieich, BRD). Unlabeled amino acids were products of Ajinomoto Co., Inc., Tokyo, Japan. Column materials were AcA 34 from LKB and DE-52 from Whatman.

Growth of Organism. Bacillus brevis (ATCC 9999) was cultivated as published by Aust & v. Döhren (1982).

Purification of Both Enzymes of Gramicidin S Synthetase. Gramicidin S synthetase was purified by a combination of procedures published by several authors (Koischwitz & Kleinkauf, 1976; Vater & Kleinkauf, 1976; Christiansen et al., 1977; Altmann et al., 1978).

Enzyme preparations were performed at 4 °C in a cold room. Fifty grams of *B. brevis* cells was thawed and suspended in 200 mL of 20 mM phosphate buffer, pH 7.2, containing 1 mM EDTA and 2 mM DTE (buffer P). The paste was passed through a French press at 4000 lb/in.^{2}, diluted with the same buffer to a volume of 400 mL, and homogenized.

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[1] Abbreviations: GS, gramicidin S synthetase (complete enzyme); GS 1, gramicidin S synthetase 1 (light enzyme, phenylalanine racemase); GS 2, gramicidin S synthetase 2 (heavy enzyme); AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; DTE, dithioerythritol; SDS, sodium dodecyl sulfate; buffer P, 20 mM phosphate buffer, pH 7.2, 1 mM EDTA, and 2 mM DTE; ΔH_{vh}, van't Hoff reaction enthalpy; EDTA, ethylenediaminetetraacetic acid.

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Table I: Purification of Gramicidin S Synthetase from *B. brevis*[a]

purification step	total protein (mg)	total act. (nmol/min)	yield (%)	sp act. [nmol min^{-1} (mg of protein)$^{-1}$]	x-fold purification
(A) Heavy Enzyme (GS 2)					
ammonium sulfate pptn	540	270	100	0.5	1.0
gel filtration on AcA 34	64	185	68	2.9	5.8
DE-52	14	101	37	7.3	14.6
sucrose gradient	3	48	18	16.0	32.0
(B) Light Enzyme, Phenylalanine Racemase (GS 1)					
ammonium sulfate pptn	360	241	100	0.67	1.0
gel filtration on AcA 34	45	189	78	4.2	6.3
DE-52	11	130	54	11.9	17.8

[a] These data are representative results of two separate purification experiments for GS 1 and GS 2. The yield is calculated on the basis of the protein obtained after the ammonium sulfate precipitation step.

The crude enzyme extract was centrifuged at 25000*g* for 20 min. In the supernatant, nucleic acids were precipitated by addition either of 0.2% poly(ethylenimine) in the presence of 0.3 M KCl or, alternatively, of 1% streptomycin sulfate. The precipitate was removed by centrifugation at 25000*g* for 20 min. In the supernatant, solid ammonium sulfate was added to a saturation of 45% and incubated for 20 min under gentle stirring. The precipitate was collected by centrifugation (20 min at 25000*g*), dissolved in 10 mL of buffer P, and applied to an ultrogel AcA 34 gel filtration column (5 × 90 cm). The protein was eluted with the same buffer at a flow rate of 100 mL/h. Both components of gramicidin S synthetase were separated completely by this procedure (Christiansen et al., 1977).

Purification of the Heavy Enzyme GS 2. GS 2 was further purified by ion-exchange chromatography on a DE-52 column (4 × 15 cm) which was equilibrated with buffer P. Most of the impurities of GS 2 were eluted by a potassium phosphate step from 20 to 150 mM. Afterwards, GS 2 was fractionated with a linear gradient from 150 to 500 mM potassium phosphate (500 mL) at a flow rate of 100 mL/h. GS 2 elutes in a conductivity range of 17–22 $m\Omega^{-1}$.

The DE-52-purified enzyme was precipitated with solid ammonium sulfate at a saturation of 55%. The precipitate was dissolved in a minimum volume of buffer P. The enzyme solution was exhaustively dialyzed against the same buffer. The concentrate was layered onto linear sucrose gradients [10–20% (w/v), total volume 34 mL] in buffer P and centrifuged at 2 °C for 42 h at 132000*g* with a Beckman SW 27 rotor.

Purification of the Light Enzyme GS 1. Phenylalanine racemase (GS 1) was also purified by ion-exchange chromatography on a DE-52 column (4 × 15 cm). The procedure was optimized as follows: The majority of the impurities in the GS 1 fraction of the AcA 34 gel filtration was removed by a step from 20 to 100 mM potassium phosphate. Afterwards, GS 1 was eluted by a linear gradient from 100 to 200 mM potassium phosphate (500 mL) at a flow rate of 100 mL/h (Figure 1A). GS 1 appears in a conductivity range of 12–15 $m\Omega^{-1}$.

A summary of the purification data for both enzymes of gramicidin S synthetase is shown in Table I. The specific activities of most of our GS 1 and GS 2 preparations were in the range of 5–10 and 8–12 nmol of gramicidin S min^{-1} (mg of protein)$^{-1}$, respectively. Occasionally also, higher rates of antibiotic formation have been obtained (see Table I). The protein concentration was estimated by the methods of Lowry et al. (1951) and Warburg & Christian (1941). The high purity of our enzyme preparations is demonstrated in Figure 1B by SDS–polyacrylamide gel electrophoresis which has been

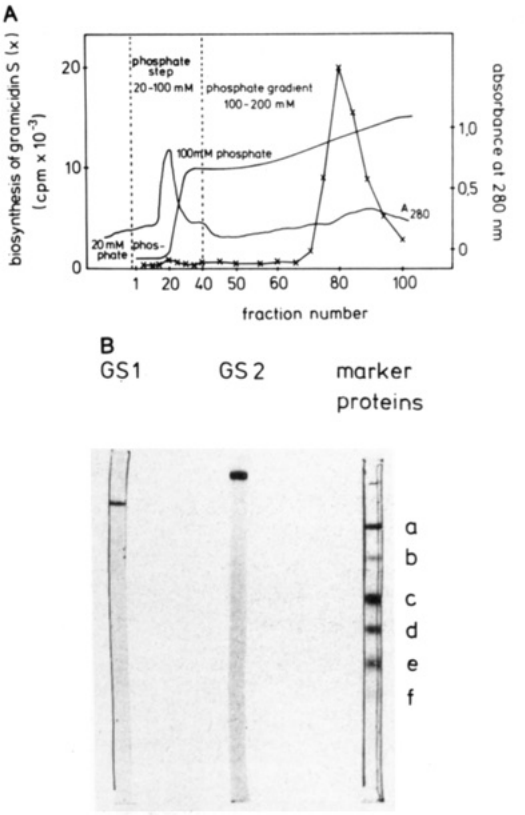

FIGURE 1: (A) DE-52 ion-exchange chromatography of GS 1. This purification step was performed as described in the text. The fractions obtained were tested for gramicidin S biosynthesis and absorbance at 280 nm. (B) SDS–polyacrylamide gels of highly purified preparations of (a) GS 1 (pool of active fractions after the DE-52 chromatography) and (b) GS 2 (pool of active fractions after sucrose gradient centrifugation). Gels containing 6% polyacrylamide and 0.13% *N,N'*-methylenebis(acrylamide) were run in 50 mM phosphate buffer, pH 7.2, in the presence of 0.5% mercaptoethanol and 0.1% SDS at room temperature. Samples were treated with 1–2% sodium dodecyl sulfate at 60 °C for 30 min. Electrophoresis was performed at 8–9 mA/tube. Marker proteins were (a) phosphorylase (94 kDa), (b) bovine serum albumin (67 kDa), (c) ovalbumin (43 kDa), (d) carbonic anhydrase (30 kDa), (e) soybean trypsin inhibitor (20.1 kDa), and (f) α-lactalbumin (14.4 kDa).

performed in a similar way as published in Vater & Kleinkauf (1976). In particular, the purification of GS 1 could be improved efficiently, requiring only two chromatography steps. This procedure is less time consuming than the technique used by Kanda et al. (1978) and yields enzyme preparations of much higher activity compared to our previously reported GS 1 purification (Vater & Kleinkauf, 1976).

Analysis of Substrate Amino Acid Activation by Gramicidin S Synthetase. (A) First Activation Step: Aminoacyl Ade-

nylate Formation. For an analysis of the aminoacyl adenylate activation processes in the biosynthesis of gramicidin S, the rate of the substrate amino acid dependent ATP–PP_i exchange reactions was measured. The experiments were performed essentially as published by Kleinkauf et al. (1969). Reaction mixtures (200 μL) contained a substrate amino acid in variable concentration, 2.5 mM ATP, 10 mM $MgCl_2$, 0.5 mM PP_i, 0.25 mM EDTA, and 1 mM DTE in 20 mM sodium phosphate buffer, pH 7.2. The exchange reaction was stopped by the addition of 0.5 mL of a Norit A mixture.

(*B*) *Second Activation Step: Thio Ester Formation.* Equilibrium binding studies of the thio ester formation of GS with substrate amino acids were performed by using techniques previously described by several authors (Kleinkauf et al., 1969; Gevers et al., 1969; Aarstad et al., 1978; Vater et al., 1982). Generally, the thiolation reactions were assayed as follows: The reaction mixtures (200 μL) were identical with those used for the ATP–PP_i exchange measurements with the exception that PP_i was omitted. Reaction times of 5–10 min were sufficient to adjust the activation equilibria at all temperatures between 5 and 37 °C. The thio ester complexes of GS were precipitated by addition of 2 mL of 5% trichloroacetic acid (w/v) and collected on membrane filters (Schleicher & Schüll; 0.45-μm pore size). The filters were washed with 5 mL of 1% trichloroacetic acid and finally with 5 mL of H_2O. Afterwards, they were dried at 110 °C for 30 min. The quantity of thio ester formed in the assay was determined by liquid scintillation counting. The data were corrected for unspecific adsorption of the ^{14}C-labeled amino acid tracers to the membrane filters by using reference samples from which the enzyme was omitted.

For the study of the effects of AMP and PP_i, the thio ester complexes of GS with ^{14}C-labeled amino acids and intermediate peptides were isolated by gel filtration on a Sephadex G-25 column (2 × 30 cm) at 3 °C. Buffer P was used as eluent and reaction medium. The reactive thio esters were incubated with various concentrations of AMP and PP_i for 30 min at 3 °C.

The remaining amount of thio ester complex was precipitated by addition of 2 mL of 5% trichloroacetic acid and assayed by using the filter technique described above.

Since thio ester complexes of GS can quantitatively be cleaved by oxidation with performic acid, thio ester analysis has been performed alternatively by the following chromatographic procedure. The protein pellets were dissolved in 100 μL of formic acid for 10 min in an ice bath; 100 μL of performic acid was added. After 2.5-h oxidation time, the performic acid was evaporated and the residue taken up in 100 μL of 0.2 M pyridine acetate buffer, pH 3.1. The extracted amino acids and peptides were separated by thin-layer chromatography and detected by radioscanning using a Berthold thin-layer scanner. For most of our experiments, however, the filter assay was applied, because the chromatographic procedures are by far more time consuming.

The Michaelis constants (K_M) for the substrate amino acid dependent ATP–PP_i exchange reactions and the dissociation constants (K_D) of the GS–substrate amino acid–thio ester complexes were determined from double-reciprocal diagrams of the concentration dependence of both activation processes. The reaction enthalpies ($\Delta H°_{vh}$) for the activation reactions of GS were derived from van't Hoff plots, ln K_M or ln K_D as a function of $1/T$. All such diagrams were evaluated by linear regression analysis of the data obtained.

For measurement of the temperature profiles of the activation reactions of GS, a Lauda RCS 20 thermostat was used.

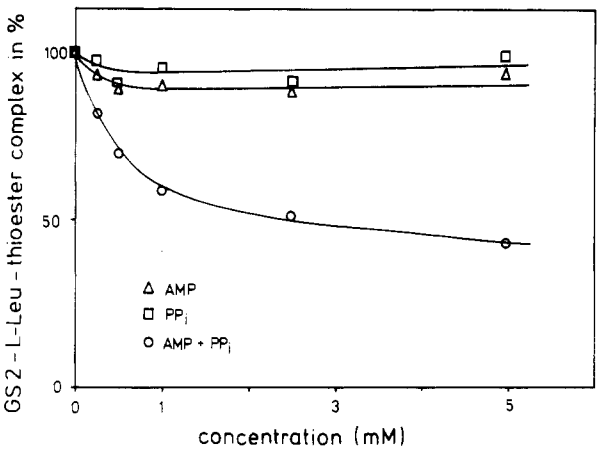

FIGURE 2: Effect of AMP (Δ), PP_i (□), and a combination of both substances (○) on the isolated thio ester complex of GS 2 with L-[^{14}C]leucine. The thio ester of GS 2 with L-[^{14}C]leucine was formed at 37 °C as indicated under Experimental Procedures. Afterwards, the complex was isolated by G-25 gel filtration. Aliquots of the active fractions were incubated with AMP and PP_i or both substances in an equimolar concentration at 3 °C for 30 min. Finally, the quantity of residual thio ester complex was measured by using the filter binding assay described in the text.

RESULTS

PP_i and AMP are byproducts of the activation reactions. Therefore, their effect on isolated thio ester complexes of GS with substrate amino acids and intermediate peptides was investigated in order to obtain further information on the reversibility of the thiolation reactions in the biosynthesis of gramicidin S. In Figure 2, it is demonstrated that the quantity of the GS 2–L-[^{14}C]leucine–thio ester complex is reduced, if both PP_i and AMP are added simultaneously to the reaction medium in a concentration of 0.1–10 mM each. PP_i and AMP alone show only weak effects. Similar results have been obtained for the thio esters of GS with the other substrate amino acids. The thio ester complexes of GS 2 with intermediate peptides, however, are stable toward these agents.

We studied the substrate amino acid activation reactions of GS at saturating ATP and Mg^{2+} concentrations. One of the prominent features of these processes is the large difference in the concentration profiles for both activation steps, as demonstrated for the valine activation in Figure 3A. The thio ester formation of L-valine is observed at rather low substrate concentrations between 10^{-8} and 10^{-5} M, whereas the valine-dependent ATP–PP_i exchange reaction appears at much higher valine concentrations in the range of 10^{-5}–10^{-2} M. For both processes, linear double-reciprocal diagrams were obtained (Figure 3B).

From the temperature dependence of the substrate amino acid specific ATP–PP_i exchange reactions, it is apparent that the Michaelis constants for these processes have negative temperature coefficients (Table II). An exception is observed for the ornithine activation. In this case, the K_M increases when the temperature is raised. From van't Hoff plots ln K_M as a function of $1/T$, the van't Hoff reaction enthalpy under standard conditions, $\Delta H°_{vh}$, has been derived for the aminoacyl adenylation steps. Such diagrams appear linear between 5 and 40 °C, as is demonstrated for the valine and ornithine activation in Figure 4. These results indicate that $\Delta H°_{vh}$ is constant in this temperature range.

The free enthalpies ($\Delta G°$) for these processes were determined from the Michaelis constants (K_M) according to the relationship

$$\Delta G° = RT \ln K_M$$

Table II: Temperature Dependence of Michaelis Constants (K_M) for Substrate Amino Acid Dependent ATP–PP_i Exchange Reactions of Gramicidin S Synthetase

temp (°C)	Michaelis constants, K_M (mM)				
	Phe	Pro	Val	Orn	Leu
5		0.95 ± 0.17	1.40 ± 0.24	0.012 ± 0.002	0.83 ± 0.21
10	0.033 ± 0.005	1.18 ± 0.14	1.16 ± 0.17	0.013 ± 0.001	0.69 ± 0.27
15	0.033 ± 0.005	0.95 ± 0.13	0.77 ± 0.12	0.016 ± 0.002	0.54 ± 0.07
20	0.025 ± 0.006	0.33 ± 0.03	0.57 ± 0.11	0.018 ± 0.001	0.41 ± 0.06
25	0.025 ± 0.005	0.29 ± 0.03	0.44 ± 0.07	0.017 ± 0.003	0.29 ± 0.02
30	0.025 ± 0.004		0.30 ± 0.08	0.020 ± 0.001	0.26 ± 0.06
37	0.021 ± 0.002	0.18 ± 0.05	0.23 ± 0.06	0.021 ± 0.002	0.22 ± 0.03

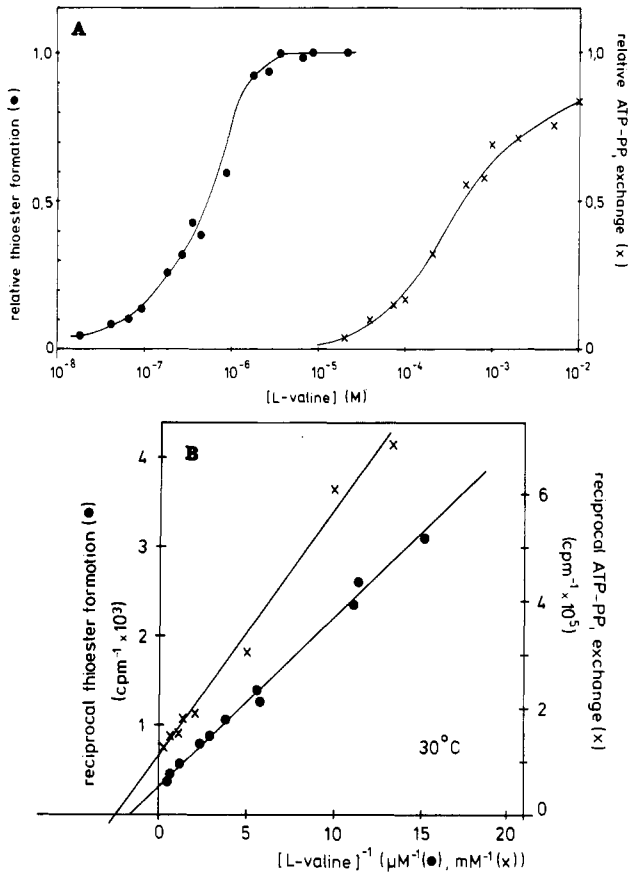

FIGURE 3: (A) Relative valine-dependent ATP–PP_i exchange (×) and relative thio ester formation of GS 2 with L-[^{14}C]valine (●) at 30 °C. Maximal thio ester incorporation of L-[^{14}C]valine into GS 2 was 9.1 pmol (100%). Reaction times were 5 min for the thio ester formation experiment and 10 min in the ATP–PP_i exchange assays. Experimental procedures were as indicated in the text. (B) Double-reciprocal plots of the concentration profiles for the valine activation reactions of GS 2. The lines were determined by linear regression analysis.

From the $\Delta G°$ and $\Delta H°$ data, the reaction entropies ($\Delta S°$) for the aminoacyl adenylate activation reactions were calculated from the Gibbs–Helmholtz equation:

$$\Delta G° = \Delta H° - T\Delta S°$$

A set of thermodynamic parameters derived for these processes at 25 °C is summarized in Table III.

The dissociation constants K_D for the thio ester complexes have been derived from linear double-reciprocal plots of thio ester formation as a function of substrate amino acid concentration (see Figure 3B). They have been determined for different temperatures. The K_D data which are in the micromolar range are summarized in Table IV. It is apparent that the dissociation constant K_D of the GS 1–Phe–thio ester complex has a positive temperature coefficient, whereas the K_D for the thio ester complexes of GS 2 with L-Pro and L-Val decreases with increasing temperature. The thio ester formation reactions of GS 2 with L-Orn and L-Leu depend only weakly on temperature.

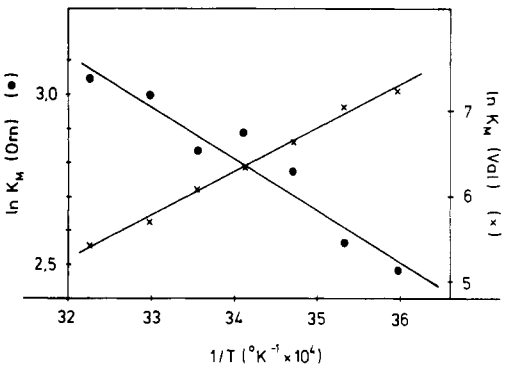

FIGURE 4: van't Hoff plots of the Michaelis constants (K_M) for the L-valine- (×) and L-ornithine- (●) dependent ATP–PP_i exchange reactions. The lines were determined by linear regression analysis.

Table III: Thermodynamic Parameters for Aminoacyl Adenylate Activation Reactions of Gramicidin S Synthetase at 25 °C[a]

amino acid	K_M (mM)[b]	$\Delta G°$ (kcal/mol)	$\Delta H°$ (kcal/mol)	$\Delta S°$ (cal mol^{-1} K^{-1})
L-Phe	0.025 ± 0.05	−6.3 ± 0.14	2.9 ± 0.5	31 ± 6
L-Pro	0.29 ± 0.03	−4.8 ± 0.08	10.8 ± 1.2	52 ± 7
L-Val	0.44 ± 0.07	−4.6 ± 0.11	10.2 ± 0.4	50 ± 3
L-Orn	0.017 ± 0.003	−6.5 ± 0.13	−3.0 ± 0.5	12 ± 2
L-Leu	0.29 ± 0.02	−4.8 ± 0.06	7.7 ± 0.6	42 ± 4

[a] The data for $\Delta G°$, $\Delta H°$, and $\Delta S°$ refer to aminoacyl adenylate formation. [b] Michaelis constants (K_M) were determined for the substrate amino acid specific ATP–PP_i exchange reactions which represent the aminoacyl adenylate equilibria.

Reaction enthalpies for the thio ester formation reactions were derived from van't Hoff diagrams of ln K_D as a function of $1/T$. On the basis of these data, a set of thermodynamic parameters (Table V) was compiled in a similar manner as described for the ATP–PP_i exchange reactions.

Discussion

In this report, we investigated the thermodynamic properties of the activation reactions in the biosynthesis of gramicidin S. The experimental material so far available implies that both aminoacyl adenylate formation and thio ester formation between gramicidin S synthetase and its substrate amino acids are reversible processes. This result is mainly based on the detection of GS-mediated specific ATP–AMP exchange reactions reported by several authors (Gevers et al., 1968, 1969; Yamada & Kurahashi, 1969; Saxholm et al., 1972). Our experiments investigating the effect of AMP and PP_i on isolated thio ester complexes of GS support these conclusions. Obviously, when both agents are added simultaneously to the reaction medium, the reverse reactions in the activation equilibria are favored. On the other hand, PP_i and AMP do not affect the thio ester complexes of the multienzyme with intermediate peptides. These results are in accordance with

Table IV: Temperature Dependence of Dissociation Constants (K_D) of the Gramicidin S Synthetase–Substrate Amino Acid–Thio Ester Complexes

temp (°C)	dissociation constants, K_D (μM)				
	Phe	Pro	Val	Orn	Leu
5	0.48 ± 0.04	1.44 ± 0.14	2.69 ± 0.18	0.35 ± 0.04	0.086 ± 0.005
10	0.57 ± 0.13	1.11 ± 0.26	2.13 ± 0.16	0.29 ± 0.03	0.088 ± 0.010
15	0.89 ± 0.11	0.83 ± 0.07	1.85 ± 0.07	0.31 ± 0.07	0.080 ± 0.008
20	1.45 ± 0.29	0.51 ± 0.03	1.00 ± 0.09	0.41 ± 0.05	0.067 ± 0.004
25	1.25 ± 0.29	0.60 ± 0.05	0.41 ± 0.12	0.35 ± 0.06	0.097 ± 0.007
30	2.26 ± 0.67	0.48 ± 0.03	0.16 ± 0.03	0.30 ± 0.06	0.079 ± 0.003
37	2.11 ± 0.72	0.44 ± 0.02	0.18 ± 0.03		

Table V: Thermodynamic Parameters for Thio Ester Activation Reactions of Gramicidin S Synthetase at 25 °C[a]

amino acid	K_D (μM)[b]	$\Delta G°$ (kcal/mol)	$\Delta H°$ (kcal/mol)	$\Delta S°$ (cal mol^{-1} K^{-1})
L-Phe	1.25 ± 0.29	−8.0 ± 0.17	−8.8 ± 0.9	−2.6 ± 0.3
L-Pro	0.60 ± 0.05	−8.5 ± 0.10	7.5 ± 1.4	54 ± 11
L-Val	0.41 ± 0.12	−8.7 ± 0.28	13.2 ± 1.6	73 ± 11
L-Orn	0.35 ± 0.06	−8.8 ± 0.13	−0.08 ± 0.77	29 ± 6
L-Leu	0.097 ± 0.007	−9.6 ± 0.08	0.91 ± 0.89	35 ± 6

[a] The data for $\Delta G°$, $\Delta H°$, and $\Delta S°$ refer to the thio ester formation. [b] K_D represents the dissociation constants of the GS–substrate amino acid–thio ester complexes which have been obtained from equilibrium binding studies, as indicated in the text.

the hypothesis that the elongation reactions in the biosynthesis of gramicidin S are irreversible, vectorial processes.

The ATP–PP_i exchange and thio ester formation reactions were analyzed as a function of the substrate amino acid concentrations at various temperatures. Saturating concentrations of ATP and Mg^{2+} were used. All experiments were performed under equilibrium conditions.

Figure 3A demonstrates the large difference in the concentration profiles for both activation steps. The thio templates can be saturated at substrate amino acid concentrations as low as 10–20 μM. Under these conditions, usually less than 10% of the aminoacyl adenylation sites are occupied. Adenylate formation and ATP–PP_i exchange become apparent at 10–100-fold higher substrate concentrations.

Linear double-reciprocal diagrams have been obtained for both types of activation reactions, as is shown in Figure 3B for the reaction of GS 2 with L-valine. The Michaelis constants derived for the ATP–PP_i exchange reactions represent the dissociation constants for the enzyme-bound aminoacyl adenylates, because there is no significant interference with the following thiolation step. The thio templates are saturated in the first turnover just at the beginning of the exchange experiment. The levels of AMP which are formed along with thio ester formation are in the submicromolar range. From the results in Figure 2, it is apparent that the reversal of thio ester formation is negligibly small at such low AMP concentrations. Therefore, the aminoacyl adenylate equilibria can be detected separately under these conditions.

The data obtained from linear double-reciprocal and Scatchard plots for the thiolation reactions represent in good approximation the dissociation constants of the GS–amino acid–thio ester complexes, because at such low concentrations, as applied in the thio ester assays, the substrate amino acids bound to the enzyme are almost quantitatively transferred to the thio templates. The reaction constants which have been determined for both activation steps at various temperatures are summarized in Tables II and IV. These results indicate that the dissociation constants for the thio ester complexes are 100–1000-fold lower than the K_M data measured for the ATP–PP_i exchange reactions in the primary aminoacyl adenylate activation. Therefore, the affinity for the substrate amino acids is much higher at the thio templates than at the adenylation centers. As a consequence of these thermodynamic features, the activation equilibria are quantitatively shifted toward thio ester formation. The thiolation sites obviously function as effective substrate pumps in the multistep process of gramicidin S biosynthesis. Thermodynamic parameters of the activation reactions of GS were determined from the temperature dependence of the K_M and K_D data. From a van't Hoff analysis of the Michaelis constants for the ATP–PP_i exchange reactions, it is apparent that the aminoacyl adenylations of Phe, Pro, Val, and Leu are endothermic processes. The ornithine activation, however, proceeds exothermically. This effect presumably depends on an additional enthalpy increment for the interaction of the δ-amino group of this substrate with reactive elements at its aminoacyl adenylation site.

The thio ester formation of GS with substrate amino acids represents the overall substrate activation at the reaction centers of this multienzyme. The van't Hoff analysis of the temperature dependence of the dissociation constants K_D for the GS–substrate amino acid–thio ester complexes shows that the thio ester formation of GS 1 with L-Phe proceeds exothermically. The overall activations of L-Pro and L-Val at GS 2 are endothermic processes, while the activation of L-Orn and L-Leu is nearly independent of temperatures between 5 and 30 °C.

The data in Tables III and V indicate that the enthalpy increments cannot account for the relatively large negative changes of the Gibbs free energy that are observed in the activation of substrate amino acids by GS 2. Here, $\Delta G°$ is predominantly controlled by the entropy contributions. In the majority of the amino acid activation reactions, the enthalpy effects that appear in the first activation step are compensated at least partially by the following thio ester formation.

An exception is provided by the thiolation and concomitant racemization of phenylalanine by GS 1. This process is mainly determined by the enthalpy increment of $\Delta G°$. In particular, the transfer of Phe from the aminoacyl adenylate reaction center of GS 1 to the thio ester site is accompanied by a relatively large exothermic effect and a marked decrease in the reaction entropy $\Delta S°$, indicating a transformation to a more ordered state.

Common to all reactive intermediates in the activation of substrate amino acids by GS are the formation of an anhydride linkage with AMP and a fixation via their amino groups at the aminoacyl adenylation sites as well as a thio ester bond at the thio templates. Such factors probably are responsible for the enthalpy increments of $\Delta G°$ in the activation reaction of GS. On the other hand, it seems obvious that the amino acid recognition and selection are mainly controlled by hydrophobic interactions between their specific side chains and complementary regions at the active sites of the enzyme. These effects essentially contribute to the entropy part of $\Delta G°$ for substrate activation.

These aspects are in accordance with a calorimetric analysis of amino acid binding to the L-isoleucine:tRNA ligase of *Escherichia coli* performed by Hinz et al. (1976). The authors found identical association enthalpies (ΔH_a) for L-Ile and a collection of its structural analogues. They demonstrated that the entropy terms (ΔS_a) are responsible for the large differences in the $\Delta G°$ of binding of these compounds.

Binding studies and kinetic experiments with substrate analogues are needed to assign the binding increments of the thermodynamic parameters to structural elements of the substrate amino acids in the biosynthesis of gramicidin S.

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Registry No. GS, 37356-20-0; L-Phe, 63-91-2; L-Pro, 147-85-3; L-Val, 72-18-4; L-Orn, 70-26-8; L-Leu, 61-90-5; phenylalanine racemase, 37290-95-2.

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Epstein–Barr Virus Induces a Unique Pyrimidine Deoxynucleoside Kinase Activity in Superinfected and Virus-Producer B Cell Lines†

Timothy Stinchcombe and Wendy Clough*

Molecular Biology Section, Department of Biological Sciences, University of Southern California, Los Angeles, California 90089

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ABSTRACT: Epstein–Barr (EB) virus induces a new pyrimidine deoxynucleoside kinase [thymidine kinase (dTk)] activity in Raji B lymphocyte cells after superinfection. This dTk activity is also present in small amounts in the HR-1 virus-producer cell line and in larger amounts in the B95-8 virus-producer line. The dTk activity induced by EB virus coelutes from DEAE-cellulose columns with deoxycytidine kinase (dCk) activity and elutes as a broad peak well separated from the large peaks of cellular dTk and dCk activities. This EB virus-induced pyrimidine deoxynucleoside kinase activity from HR-1 cells differs from cellular kinases in most basic biochemical properties but shares certain properties with the herpes simplex virus dTk.

Herpes viruses induce most of the enzymes required for replication of viral DNA in productively infected cells. These viral enzymes include DNA polymerase (Weissbach et al., 1973; Boezi et al., 1974; Huang, 1975; Allen et al., 1977; Goodman et al., 1978), DNase (Hoffman & Cheng, 1978; Clough, 1979, 1980; Hoffman, 1981), and a recently reported topoisomerase (Biswal et al., 1983). Virus-induced pyrimidine deoxynucleoside kinase [thymidine kinase (dTk)][1] activity has been detected in cells productively infected with a variety of herpes viruses, including herpes simplex virus (Ogino et al., 1973; Jamieson & Subak-Sharpe, 1974), varicella-zoster virus (Ogino et al., 1977), and herpes virus saimiri (Honess et al.,

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[1] Abbreviations: EB virus, Epstein–Barr virus; dTk, thymidine kinase; dCk, deoxycytidine kinase; dT, thymidine; dC, deoxycytidine; TPA, 12-*O*-tetradecanoylphorbal 13-acetate; dTk$^-$, dTk negative; DEAE, diethylaminoethyl; PMSF, phenylmethanesulfonyl fluoride; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; K_2PO_4, mixture of mono- and dipotassium phosphate salts adjusted to pH 8.0.

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