Identification of Two Enzymes Responsible for the Synthesis of the Initial Portion of Linear Gramicidin†

Hugh A. Akers,‡ Sung G. Lee, and Fritz Lipmann*

ABSTRACT: Linear gramicidin is an antibiotic produced by *Bacillus brevis* (ATCC 8185) and has the following structure: *N*-formyl-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-ethanolamine. In previous work on its cell-free synthesis, an enzyme-bound pentadecapeptide was isolated which, after chemical aminoethanolysis and formylation, proved to be identical with authentic linear gramicidin (Bauer, K., et al. (1972), *Biochemistry 11,* 3266). The *B. brevis* used in these experiments produces tyrocidine in addition to linear gramicidin. The amino acid content of these two antibiotics overlaps except for the unique presence of glycine and alanine in linear gramicidin. This paper describes the results of examining the *B. brevis* extracts for activation of these two amino acids and led to the isolation of two polyenzymes, designated glycine enzyme and alanine enzyme, that synthesize the initial peptide portion of linear gramicidin. It was found that a purified preparation of the former activates L-valine and glycine and of the latter activates L-alanine, L-leucine, and L-valine. Molecular weights for the two enzymes were estimated from sucrose density gradient centrifugation to be 160 000 for the glycine enzyme and 350 000 for the alanine enzyme. Each enzyme was found to contain 1 mol of 4′-phosphopantetheine. The activated amino acids were shown to be bound to the two polyenzymes in thioester linkage. When both L-valine and glycine were supplied to the glycine enzyme, little valine-glycine was bound, but large amounts of free L-valylglycine and smaller amounts of L-Val-Gly-diketopiperazine were formed depending on addition of ATP. The ATP-PP_i exchange, synthetic activities, thioester formation, and molecular size indicate that the glycine enzyme produces the initiating amino acids L-Val-Gly and the alanine enzyme the next, pentapeptide sequence, L-Ala-D-Leu-L-Ala-D-Val-L-Val. Because the alanine enzyme does not activate D-valine, and poorly if at all, D-leucine, presumably the D amino acids are formed from the L enantiomers by enzymatic racemization. Experiments with the initial linear gramicidin constituent amino acids and the mixture of the glycine and alanine enzymes suggest the two enzymes are responsible for biosynthesis to the heptapeptide stage and cause sequential addition of the first seven amino acids in a manner analogous to that found in the previously studied synthesis of tyrocidine and gramicidin S (Lipmann, F. (1973), *Acc. Chem. Res. 6,* 361).

The Dubos strain of *Bacillus brevis* (ATCC 8185) produces a mixture of two antibiotics consisting of larger amounts of tyrocidine and smaller amounts of linear gramicidin in a ratio of 5–10:1 according to Hotchkiss (1944). The relatively small quantity of linear gramicidin produced makes its biosynthesis difficult to analyze, whereas we have been able to resolve extensively the tyrocidine synthesis system (Lipmann, 1973). An additional difficulty in investigating linear gramicidin synthesis is that only two of its amino acids are not present in tyrocidine, namely, glycine and L-alanine (Figure 1), and Roskoski et al. (1970a) have observed that both of these will bind to crude *B. brevis* extracts in thioester linkage. This fact, together with the finding by Bauer et al. (1972) that the pentadecapeptide chain of linear gramicidin can be synthesized in crude extracts and will remain enzyme-bound in thioester linkage, makes it clear that the mechanism of synthesis should be the same for both antibiotics.

In the earlier paper on linear gramicidin, Bauer et al. (1972) aimed at using the crude extract for total in vitro synthesis; this they achieved by a combination of biosynthesis and chemical synthesis, but they did not attempt to isolate the polyenzymes that presumably participate in its biosynthesis. In the study reported here, we used the presence of glycine and alanine in the early sequence of linear gramicidin as a guide to isolating the enzymes that synthesize its initial portion. Beginning with Sephadex G-200 chromatography, we isolated fractions that activate glycine or alanine and called them the glycine enzyme and alanine enzyme. The former activates the first two amino acids, valine and glycine, and initiates the chain; the latter activates the following five-amino acid sequence, L-Ala-D-Leu-L-Ala-D-Val-L-Val. In confirmation of the work by Bauer et al. (1972), we found that formylation apparently occurs after chain initiation, since *N*-formylvaline was not a substrate for the glycine enzyme. A recent report from Kurahashi's group (Akashi et al., 1977) describes the formyl transfer to enzyme-bound valine.

Both enzymes were found to contain 4′-phosphopantetheine, completing the analogy between the mechanism of biosynthesis of the two antibiotics synthesized by this *B. brevis* strain. A preliminary account of this work has been presented (Akers et al., 1976).

Methods

Amino acid activation, measured by amino acid dependent ATP-PP_i[1] exchange, was carried out as described by Lee and Lipmann (1975) and was used to locate the enzyme fractions. Polyacrylamide gel electrophoresis was performed as in Lee et al. (1973); to locate further the glycine and alanine enzymes,

† From the Rockefeller University, New York, New York 10021. *Received June 27, 1977.* Supported by Grant GM-13972 (F.L.) and fellowship IF22 AI03034 from the National Institutes of Health (H.A.), and by Grant PCM 75-21165 (S.G.L.) from the National Science Foundation.

‡ Present address: Department of Chemistry, Lamar University, Beaumont, Texas 77710.

[1] Abbreviations used: PP_i, inorganic pyrophosphate; DNP, dinitrophenyl; DEAE, diethylaminoethyl.

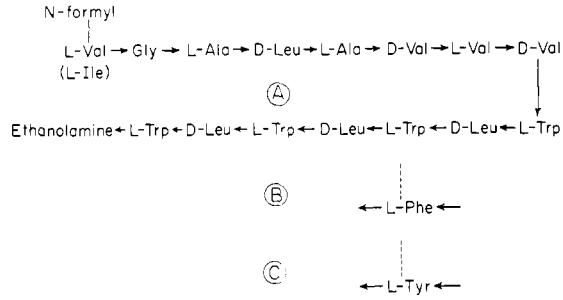

FIGURE 1: Linear gramicidin (A, B, and C).

the frozen gel was sliced and the slices were assayed for amino acid dependent ATP-PP_i exchange.

Cellulose thin-layer chromatography with 2-butanol:2-butanone:dicyclohexylamine:water (10:10:2:5) as a solvent system was used to resolve valine, glycine, valylglycine, and L-Val-Gly-diketopiperazine. Dipeptides, diketopiperazines, and dinitrophenyl derivatives were identified by cellulose and/or silica gel thin-layer chromatography using the following solvent systems: chloroform:isoamyl alcohol:acetic acid (70:30:3); diethyl ether:acetic acid:water (100:1:1); and 1-butanol:acetic acid:water (4:1:1).

Labeled compounds were located on thin-layer chromatograms and electropherograms by radioautography, scanning, or liquid scintillation spectrometry of segments. Amino acids and dipeptides were located by ninhydrin and diketopiperazines by a *tert*-butyl hypochlorite starch–iodide spray (Mazer et al., 1962).

Thioester-linked amino acids and enzyme-bound peptides were preliminarily determined in the trichloroacetic acid precipitate described by Lee et al. (1973) and were more thoroughly identified, as described below, through their release by hydroxylamine, alkali, and performic acid oxidation; omission of ATP from one sample in each assay provided the blank. The counting efficiency was usually 15–35% for the radioactive amino acids in the precipitate collected on Millipore filters and was determined by drying a known number of dpm of L-[^{14}C]alanine on duplicate protein precipitates. All samples were washed with the respective unlabeled 1 mM amino acid solutions. The thioester link to the protein was confirmed by application of specific tests as described in the text.

Pantothenic acid was liberated from protein-bound 4′-phosphopantetheine and determined microbiologically with *Lactobacillus plantarum* (ATCC 8014) by the method of Pugh and Wakil (1965). For this purpose, polyacrylamide gel electrophoresis segments were macerated with a spatula in 1 N KOH and heated in a steam bath for 1 h to separate 4′-phosphopantetheine from the protein. The filtrate was then adjusted to pH 8 with HCl and exposed to alkaline phosphatase (Sigma) for 2 h at 37 °C to liberate free pantothenate for the microbiological assay.

The optical configuration of labeled valine was determined by exposure for 30 min at 37 °C to L-amino acid oxidase (Worthington), with 10 μg of oxidase, 10 μg of catalase, and 100 mM potassium phosphate at pH 7.5, in a total volume of 0.1 mL. The α-ketoisovalerate was separated from D-valine as the 2,3-dinitrophenylhydrazine conjugate at neutral pH by extraction with diethyl ether.

Experiments using purified enzymes were normally conducted with enzymes purified at least through the third purification step (see Table I). Because of the large amount of enzymes required for the experiment investigating the glycine enzyme products, enzyme from the second purification step was used. The tyrocidine biosynthetic enzymes had been removed sufficiently by the second purification step to permit this substitution without interference.

TABLE I: Purification of Glycine and Alanine Polyenzymes.[a]

Purification step	Protein (mg)[b]	Amino acid dependent ATP-PP_i exchange (cpm)	Sp act. (cpm/mg of (protein)	Yield (%)
	Glycine Dependent			
Glycine enzyme				
1. Sephadex G-200	825	2.6×10^9	3.1×10^6	100
2. DEAE-cellulose	42	1.1×10^9	2.6×10^7	42
3. Hydroxylapatite	17	8.2×10^8	4.8×10^7	32
4. Sucrose gradient	4	4.7×10^8	1.2×10^8	18
	Alanine Dependent			
Alanine enzyme				
1. Sephadex G-200	975	2.3×10^9	2.4×10^6	100
2. DEAE-cellulose 1	85	1.5×10^9	1.8×10^7	65
3. DEAE-cellulose 2	35	1.0×10^9	2.9×10^7	43
4. Sucrose gradient	12	4.9×10^8	4.0×10^7	21

[a] A 30 000*g* supernatant containing 17.5 g of protein was prepared from 150 g of cells (Lee et al., 1973). The ammonium sulfate (33–50% saturation) fraction of this supernatant containing 5.2 g of protein was applied to gel filtration. Yields were calculated relative to the fractions recovered from Sephadex G-200 gel filtration. [b] Protein determinations were by the method of Lowry et al. (1951) for steps 1 and 2, and by biuret (Zamenhof, 1957) for steps 3 and 4.

Materials. All chemicals were from Aldrich Chemical Co. or Sigma Chemical Co. except L-Val-Gly-diketopiperazine which was a gift from Dr. K. D. Kopple, and dinitrophenyl-L-valylglycine (DNP-L-Val-Gly) which was prepared from 1-fluoro-2,4-dinitrobenzene and L-valylglycine (Porter, 1957).

Results

Purification of the Glycine- and Alanine-Activating Fractions. B. brevis cells (150 g) harvested near the peak of β-alanine uptake (Lee et al., 1975) were treated with lysozyme and DNase for 10 min at 37 °C (Lee et al., 1973), followed by centrifugation at 4 °C for 15 min at 20 000*g* which produced a crude extract. All subsequent purification procedures were likewise carried out at 4 °C. The fraction of the crude extract that precipitated between 33% and 50% ammonium sulfate saturation was dissolved and subjected to Sephadex G-200 chromatography as shown in Figure 2 and described in detail in the legend. The positions of the glycine and alanine activation peaks in the Sephadex filtrate were located by assaying for ATP-PP_i exchange. To obtain markers, the activation peaks of phenylalanine, proline, and ornithine, known from the work on tyrocidine synthesis, were used as indicators for the position of the phenylalanine-activating enzyme, the intermediate enzyme, and the heavy enzyme of tyrocidine synthesis with molecular weights of 100 000, 230 000, and 440 000, respectively, which were present in this Sephadex G-200 filtrate (Lee et al., 1973; Roskoski et al., 1970a). By comparision,

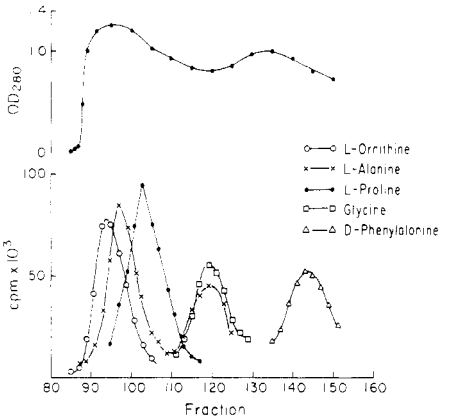

FIGURE 2: Separation of the alanine and glycine enzymes by Sephadex G-200 gel filtration. About 2.6 g of protein from the 33–50% ammonium sulfate fraction of the crude extract was applied to a Sephadex G-200 column (9.5 × 85 cm). Elution was with 0.02 M triethanolamine buffer, pH 7.7, containing 0.5 mM EDTA, 1 mM dithiothreitol, and 0.1 M KCl. The 27-mL fractions were assayed for ATP-PP_i exchange dependent upon L-alanine and glycine in order to locate two of the linear gramicidin biosynthetic enzymes, while their location was compared with ATP-PP_i exchange dependent on L-ornithine, L-proline, and D-phenylalanine which indicated the sites of the heavy, intermediate, and light tyrocidine biosynthetic enzymes.

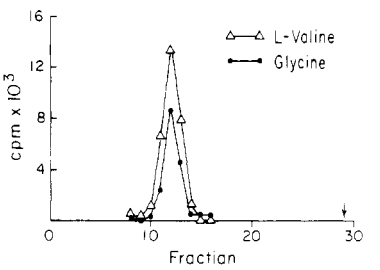

FIGURE 3: Final purification of the glycine enzyme by sucrose gradient centrifugation. About 100 μg (as much as 1 mg in other runs) of glycine enzyme from hydroxylapatite in 0.2 mL of buffer B with 0.2 M KCl was layered on a 13.5-mL gradient between 5% sucrose with 0.2 M KCl in buffer B and 30% sucrose with 0.1 M KCl in buffer B and was centrifuged in the Spinco for 18 h at 40 000 rpm using a SW-40 rotor. The gradient was fractionated by a gravity-fed 40% sucrose solution applied to the bottom (arrow at right of figure) while fractions (29 total) were collected from the top. The ATP-PP_i exchange assay dependent on glycine and L-valine was performed with 5-μL aliquots of the fractions. No L-alanine-dependent exchange was observed.

an already rather fair estimate was obtained regarding the position of the alanine- and glycine-activating peaks. In the Sephadex chromatogram of Figure 2, the high alanine peak stands out clearly, situated between the intermediate and heavy tyrocidine enzyme peaks. The glycine peak falls into the interval between the positions of the phenylalanine-activating and intermediate enzymes; it overlaps with a smaller alanine peak which appears to be due to a fragment of the alanine enzyme that easily decomposes into subfractions as observed by Lee et al. (1973) with tyrocidine synthesizing enzymes. In contrast, the glycine enzyme proved to be quite stable.

Purification of the Glycine Enzyme. The glycine enzyme, positioned in the Sephadex G-200 chromatogram between fractions 116 and 124, was applied to a DEAE-cellulose column (4 × 15 cm) previously equilibrated with buffer B of Lee et al. (1973) containing 0.1 M KCl and 5% sucrose. Elution was performed with a linear KCl (0.1–0.4 M) gradient in a mixture of buffer B and 5% sucrose. Fractions containing ATP-PP_i exchange activities dependent upon glycine were concentrated with a Diaflo apparatus.

The glycine enzyme fraction from DEAE-cellulose was equilibrated by Diaflo with 5 mM phosphate buffer (pH 7.0) containing 5% sucrose and 1 mM dithiothreitol and was applied to a hydroxylapatite column (1.8 × 45 cm) previously equilibrated with the same buffer. Elution was by means of a phosphate gradient (5–150 mM, pH 7.0) containing 5% sucrose and 1 mM dithiothreitol. The fractions with exchange activity dependent on glycine were pooled and concentrated by the Diaflo apparatus.

Up to 1 mg of the glycine enzyme fraction from hydroxylapatite in 0.2 mL of buffer B containing 0.2 M KCl was layered on a 13.5-mL gradient between 5% sucrose with 0.2 M KCl in buffer B and 30% sucrose with 0.1 M KCl in buffer B; it was then centrifuged for 18 h at 40 000 rpm in a Spinco centrifuge using a SW-40 rotor. To fractionate the gradient, a syringe needle was inserted into the bottom of the tube and 40% sucrose was forced through the needle, which was connected by means of a $\frac{1}{16}$-in. diameter tube to a reservoir of 40% sucrose placed 2 yards above the gradient tube. Fractions were collected from the top by allowing the gradient to flow, through an outlet placed in a stopper that sealed the top of the tube, at a rate of 2 drops per min. The resulting fractions were assayed for exchange activity dependent on glycine or L-valine (Figure 3). The degree of purification of the glycine enzyme after sucrose gradient centrifugation is shown in Table I; as seen in Figure 3, this enzyme activates valine and glycine, the first two amino acids in linear gramicidin.

Purification of the Alanine Enzyme. Because the high peak for alanine in gel filtration (Figure 2) lies between the 440 000 heavy (activating 6 amino acids) and the 230 000 intermediate (activating 3 amino acids) polyenzymes of tyrocidine synthesis, it seemed likely that it would activate two alanines of linear gramicidin since they are the third and fifth amino acids in sequence after valine-glycine. The position of the alanine peak also indicated it would be a polyenzyme that activates less than six and more than three amino acids.

The early peak in Figure 2 (fractions 96–101) that eluted from Sephadex G-200 gel filtration and showed alanine-dependent ATP-PP_i exchange was chromatographed further on DEAE-cellulose by the procedures described for the glycine enzyme. As shown in Figure 2, the alanine enzyme dissociated on Sephadex G-200 as well as on hydroxylapatite (not shown) into lower molecular weight components. To further separate the alanine enzyme from the tyrocidine synthesizing enzymes, it was subjected to a second chromatography on DEAE-cellulose. For this purpose, the KCl concentration of the eluate from the first DEAE-cellulose chromatogram was reduced to 0.1 M by dialysis, and the sample was reapplied to a DEAE-cellulose column (1.2 × 10 cm). This second column was equilibrated and eluted as described. The final purification step by sucrose density gradient using procedures analogous to those described for the glycine enzyme is shown in Figure 4 and Table I. The alanine enzyme dissociated in all procedures tested into a slower sedimenting component which appeared in fractions corresponding to molecular weights 40–50% that of the faster sedimenting form. Occasionally, when smaller fractions were taken as in Figure 5, it was found they separated into three components during sucrose gradient centrifugation. Yields from 150 g of cells are recorded in Table I using as reference the pooled glycine or alanine enzymes recovered from the Sephadex G-200 filtrate.

Molecular Weight Determination by Sucrose Gradient Centrifugation. Sedimentation coefficients of the alanine and glycine enzymes were measured by sucrose density gradient centrifugation (Figure 5). Values of 14.1 S and 8.3 S were

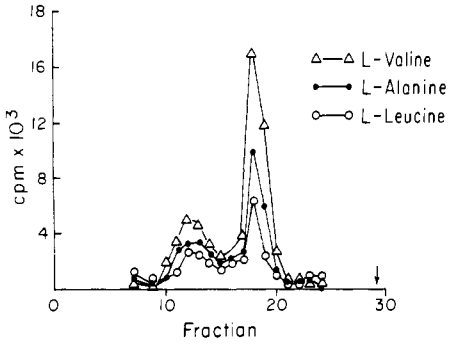

FIGURE 4: Purification of alanine enzyme by sucrose gradient centrifugation. About 120 μg (as much as 1 mg in other runs) of alanine enzyme from the second DEAE-cellulose step in 0.2 mL of buffer B with 0.2 M KCl was layered on a 13.5-mL gradient between 5% sucrose with 0.2 M KCl in buffer B and 30% sucrose with 0.1 M KCl in buffer B and was centrifuged for 18 h in the Spinco at 40 000 rpm using a SW-40 rotor. The gradient was fractionated by a gravity-fed 40% sucrose solution applied to the bottom (arrow right of Figure) while fractions (29 total) were collected from the top. The ATP-PP_i exchange assay dependent on L-alanine, L-leucine, and L-valine was performed with 5-μL aliquots of the fractions. No glycine-dependent exchange was observed. The slower moving activity presumably represents decomposition products of the alanine enzyme, seemingly composed of two subunits since it sediments to the same position as the glycine enzyme (cf. Figure 3).

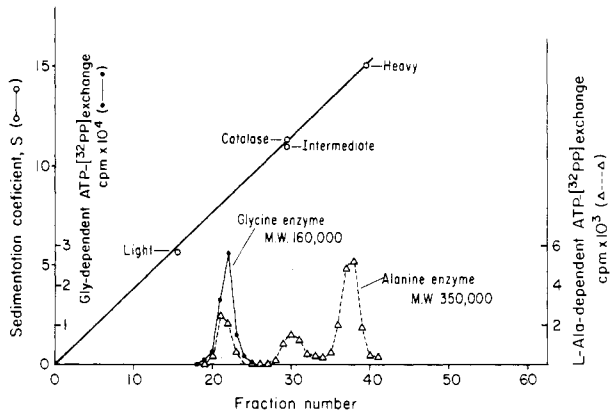

FIGURE 5: Molecular weight estimation of the glycine and alanine enzymes by sucrose gradient centrifugation. Purified light, intermediate, and heavy tyrocidine biosynthetic polyenzymes (Lee et al., 1973) and catalase were used as molecular weight standards (about 5–10 μg of each protein). The standards, together with the glycine and alanine enzymes (about 5 μg of each from sucrose gradient centrifugation) in 0.1 mL of buffer B with 0.2 M KCl, were layered on a 13.5-mL gradient between 5% sucrose with 0.2 M KCl in buffer B and 30% sucrose with 0.1 M KCl in buffer B and were centrifuged for 18 h at 40 000 rpm in the Spinco using a SW-40 rotor. The gradient was fractionated by a gravity-fed 40% sucrose solution applied to the bottom (left of figure) while 64 fractions were collected from the top. The tyrocidine biosynthetic polyenzymes were assayed in 5-μL aliquots by ATP-PP_i exchange dependent upon D-phenylalanine (light), L-proline (intermediate), and L-ornithine (heavy). Catalase was assayed spectrophotometrically (Beers and Sizer, 1952). Glycine and alanine enzymes were detected by exchange activities dependent upon glycine and L-alanine, respectively. The two alanine enzyme breakdown products were calculated to have molecular weights of 146 000 (7.9 S) and 250 000 (11.3 S).

found. Standards consisted of catalase, as well as the light, intermediate and heavy tyrocidine biosynthetic polyenzymes which had sedimentation coefficients of 11.3, 5.7, 11, and 15 S, respectively (Roskoski et al., 1970a). Molecular weights of 160 000 and 350 000 were calculated for the glycine and alanine enzymes (Martin and Ames, 1961) using a value of 250 000 for catalase (Kiseler et al., 1967).

4′-Phosphopantetheine Content of the Glycine and Alanine Enzymes. The amino acid dependent ATP-PP_i exchange on

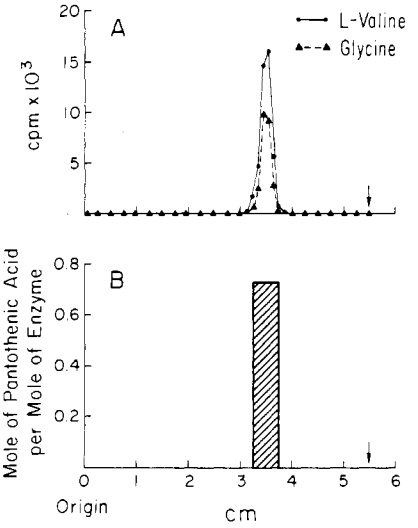

FIGURE 6: Gel electrophoresis of the glycine enzyme. Enzyme from sucrose gradient centrifugation was electrophoresed on 5% polyacrylamide gels with 0.02 M triethanolamine buffer, pH 8.0. The gels were then segmented and assayed microbiologically for pantetheine, while identical gels were sliced and assayed for amino acid-dependent ATP-PP_i exchange and stained for protein with Coomassie brilliant blue (Sigma). Greater than 75% of the protein was found to be coincident with the peak of enzyme activity. Dimensions are relative to the origin; a bromophenol blue dye front was at 5.5 cm. (A) To locate the glycine enzyme, gel slices were assayed for glycine and L-valine dependent on ATP-PP_i exchange. No ATP-PP_i exchange dependent on L-alanine, L-ornithine, L-proline, or D-phenylalanine was observed in the 3–4-cm region. (B) A gel containing 20 μg of glycine enzyme was cut at 3.25 and 3.75 cm and pantetheine determinations were made on the three resulting segments.

portions of 1-mm slices of polyacrylamide gels following electrophoresis is shown in Figure 6A for the glycine enzyme and Figure 7A for the alanine enzyme. Gels were stained for protein with Coomassie brilliant blue R (Sigma) (not shown in the figures); greater than 75% of the protein in each gel was coincident with the peak of amino acid dependent ATP-PP_i exchange activity. Another gel (Figures 6B and 7B) for each enzyme was sliced in larger segments and assayed microbiologically for pantothenate as described in Methods. Both enzymes were found to contain pantothenate, presumably bound to them as the 4′-phosphopantetheine because both alkali and alkaline phosphatase were required to release it. A peptidase was not needed for the liberation of pantothenate (Pugh and Wakil, 1965) when an impure alkaline phosphatase preparation, presumably containing a peptidase, was used. The values given in Figures 6B and 7B are calculated assuming the complete release and recovery of pantothenate from a pure enzyme. It is suggested, therefore, that each enzyme contains 1 mol of 4′-phosphopantetheine per mol of enzyme.

Experiments with the Purified Glycine Enzyme. The ATP-PP_i exchange with various amino acids using the hydroxylapatite fraction (step 3 in Table I) is shown in Table II. The most abundant exchange was found dependent on (section 1) L-valine and glycine. None was found with *N*-formyl-DL-valine, further confirming that the formylation occurs after valine incorporation (Bauer et al., 1972). As was seen with tyrocidine synthesis (Lee et al., 1973), the exchange figures vary for the different amino acids due, probably, to the variability of their affinities. Minimal ATP-PP_i exchange was found to be dependent on D-valine and L-alanine, but somewhat more on L-leucine, indicating low contamination of the glycine enzyme with components of the sequence synthesized by the alanine enzyme (Table II, section 2). The very small ATP-PP_i exchange with phenylalanine, tryptophan, ornithine,

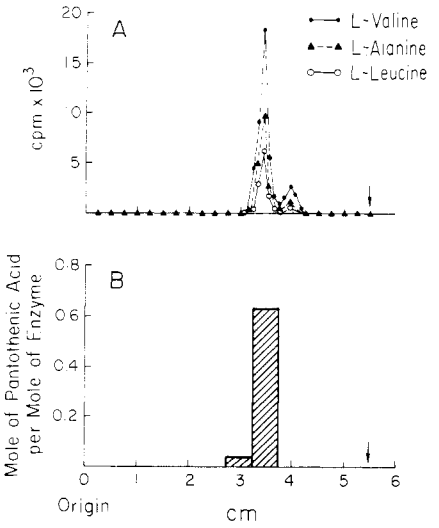

FIGURE 7: Gel electrophoresis of the alanine enzyme. Enzyme from sucrose gradient centrifugation was electrophoresed on 5% polyacrylamide gels with 0.02 M triethanolamine buffer, pH 8.0. The gels were then segmented and assayed microbiologically for pantetheine, while additional gels were sliced and assayed for amino acid dependent ATP-PP_i exchange and stained for protein with Coomassie blue. Greater than 75% of the protein was found to be coincident with the main peak of enzyme activity. Dimensions are relative to the origin; a bromophenol blue dye front was at 5.5 cm, indicated by the arrow. (A) To locate the alanine enzyme, gel slices were assayed for ATP-PP_i exchange dependent on L-alanine, L-valine, and L-leucine. Two peaks of enzyme activity were found; the smaller peak was presumably a decomposition form of the larger one. No ATP-PP_i exchange dependent on L-proline, L-ornithine, L-tryptophan, or glycine was observed in the 3–4-cm region. (B) A gel containing 35 μg of alanine enzyme was cut at 2.75, 3.25, 3.75, and 4.25 cm. Pantetheine determinations were made on the resulting five segments. The majority of the pantetheine was present in a segment that coincided with the peak of alanine enzyme activity. A small amount of pantetheine was found in the 2.75–3.25-cm segment which corresponded to the side of the peak of enzyme activity. No pantetheine was found in the smaller alanine enzyme peak.

TABLE II: Amino Acid Dependent ATP-$^{32}PP_i$ Exchange with the Glycine Enzyme.

No.	Amino acids	[^{32}P]ATP formed[a] (nmol)
1.	L-Valine	79.3
	N-Formyl-DL-valine	0.1
	L-Isoleucine	34.9
	Glycine	39.1
2.	L-Alanine	3.6
	L-Leucine	7.1
	D-Valine	4.9
3.	L-Phenylalanine	1.8
	L-Tryptophan	3.4
	L-Ornithine	1.1
	L-Proline	0.9

[a] Assayed as described in Methods using 6.4 μg of protein from the hydroxylapatite step (see Table I).

and proline, all components of tyrocidine (Table II, section 2), indicated little contamination by tyrocidine synthesizing enzymes. The glycine enzyme actively catalyzes an ATP-PP_i exchange dependent upon L-isoleucine (Table II). The latter is to be expected considering the frequent substitution of the first L-valine in linear gramicidin by L-isoleucine (Sarges and Witkop, 1965).

The binding of amino acids in thioester linkage is presented for the glycine enzyme in Table III. This binding increased proportionally with increasing concentrations of glycine and

TABLE III: Binding of Amino Acids to Glycine Enzyme in Thioester Linkage.[a]

Labeled amino acid	Amino acid concn (mM)	Moles of amino acid bound/mol of Gly enzyme[b]
L-Valine	0.10	0.14
	0.25	0.28
	0.50	0.40
	0.75	0.48
Glycine	0.10	0.10
	0.25	0.23
	0.50	0.36
	0.75	0.45
L-Isoleucine	0.10	0.05
L-Alanine	0.10	0.01
L-Ornithine	0.10	0.01

[a] Amino acids bound to the enzyme were determined in the trichloroacetic acid precipitate described by Lee et al. (1973). Incubations (final pH 7.6) contained 10 Ci/mol of ^{14}C-labeled amino acid. Corrections were made for a blank value determined by the omission of ATP from a duplicate L-[^{14}C]valine experiment. [b] Enzyme was from hydroxylapatite (100 μg/assay). In these calculations, we assume the enzyme to be 75% pure based on acrylamide gel electrophoresis (cf. Figure 6).

of L-valine, with a ratio of bound glycine to valine of nearly 1:1 over the concentration range examined. The less than stoichiometric binding of labeled amino acids to enzyme is consistent with observations made with the tyrocidine enzymes (Lee et al., 1973). The binding of L-valine in thioester linkage to the glycine enzyme was not affected by the presence of *N*-formyl-DL-valine, again indicating that formylation does not occur prior to the linking of valine to the enzyme. This bears out Bauer et al. (1972) who observed that unformylated enzyme-bound pentadecapeptide may be formed by an ammonium sulfate fraction of the enzyme mixture. With crude bacterial extracts and added formyl donor, however, evidence for the formylation of enzyme-bound pentadecapeptide was obtained. In the latter case, the only missing reaction was the transfer of ethanolamine to the carboxyl terminal which we were unable to obtain except by chemical means (Bauer et al., 1972). The incorporation of [^{14}C]serine into the ethanolamine of linear gramicidin in live *B. brevis* (ATCC 8185) has been reported recently by Kubota and Kagawa (1976).

The Condensation of Valine and Glycine on the Glycine Enzyme. Glycine enzyme from hydroxylapatite (1 mg) was incubated for 30 min at 37 °C in a medium composed of 50 mM triethanolamine, pH 7.8, 20 mM magnesium acetate, 5 mM KCl, 1 mM dithiothreitol, and 4 mM ATP containing 0.1 mM L-[^{3}H]valine (10 μCi) or [^{3}H]glycine, in a total volume of 1 mL. The protein was lyophilized after separation from low molecular weight material on a Sephadex G-50 column (2 × 45 cm; 5 mM phosphate, pH 6.8, as eluent). Portions of the reaction mixture were treated for 10 min at 37 °C with 2 M hydroxylamine (salt-free) at pH 7.8, for 10 min at 37 ° C with HCl to pH 2.4, for 10 min at 37 °C with KOH to pH 13, and with performic acid (Frøshov et al., 1970). Trichloroacetic acid precipitates were compared before and after the above treatments by liquid scintillation spectrometry. The protein-bound glycine and valine were found to be stable to acid but labile to KOH, hydroxylamine, and performic acid. This is consistent with a thioester linkage between amino acids and enzyme (Gevers et al., 1969; Frøshov et al., 1970). On cellulose thin-

TABLE IV: Amino Acid Dependent ATP-$^{32}PP_i$ Exchange with the Alanine Enzyme.

No.	Amino acid	[^{32}P]ATP formed[a] (nmol)
1.	L-Alanine	12.2
	L-Valine	22.8
	D-Valine	0.4
	L-Leucine	8.8
	D-Leucine	2.2
2.	Glycine	0.6
3.	L-Phenylalanine	1.1
	L-Tryptophan	0.7
	L-Ornithine	0.1
	L-Proline	0.6
	L-Isoleucine	1.8

[a] Assayed as described in Methods using 3.7 μg of protein from the sucrose density gradient step.

TABLE V: Binding of Amino Acids to Alanine Enzyme in Thioester Linkage.[a]

Labeled amino acid	Amino acid concn (mM)	Moles of amino acid bound/mol of Ala enzyme[b]
L-Valine	0.10	0.16
	0.25	0.46
	0.75	0.84
L-Alanine	0.10	0.13
	0.25	0.38
	0.75	0.77
L-Leucine	0.10	0.07
	0.25	0.21
	0.75	0.45
Glycine	0.10	0.01
L-Ornithine	0.10	0.01

[a] Amino acids bound to the enzyme were determined in the trichloroacetic acid precipitate described by Lee et al. (1973). Incubations (final pH 7.6) contained 50 Ci/mol of ^{14}C-labeled amino acid. Corrections were made for a blank value determined by the omission of ATP from a duplicate [^{14}C]valine experiment. [b] Enzyme was from second DEAE-cellulose (330 μg/assay). In these calculations, we assume an enzyme purity of 75% based on acrylamide gel electrophoresis (cf. Figure 7).

layer chromatography, the tritium released by performic acid from a trichloroacetic acid precipitate comigrated with valine or glycine in the respective experiments.

Glycine Enzyme Products on Addition of a Combination of Valine and Glycine. Glycine enzyme (2 mg) from DEAE-cellulose was incubated for 30 min at 37 °C in 1 mL of the same medium as used in the experiment described in the preceding paragraph containing 5 μCi of L-[^{14}C]valine (0.1 mM) and 25 μCi of [^{3}H]glycine (0.1 mM). The labeled amino acids were first purified by cellulose thin-layer chromatography. The pH of the incubation mixture was adjusted to 7.4 with normal KOH, and the reaction was terminated by application to a Sephadex G-50 column (2 × 45 cm; 5 mM phosphate, pH 6.8, as eluent). Two fractions were produced, the first containing protein and the second composed of low molecular weight material; they were both lyophilized. The protein fraction was also treated with performic acid (Frøshov et al., 1970) followed by lyophilization. The two fractions were then extracted with butanol:chloroform (4:1), and the extracts were reduced to a small volume and subjected to cellulose thin-layer chromatography. The excess of added glycine (R_f 0.25) and valine (R_f 0.45) appeared in the chromatogram. In addition, L-valylglycine (R_f 0.75) and L-Val-Gly-diketopiperazine were found in the low molecular weight region from the G-50 column. However, in the organic extracts of the protein fraction treated with performic acid, only L-valylglycine and small amounts of valine and glycine were present.

L-Valylglycine and L-Val-Gly-diketopiperazine were identified by their electrophoretic behavior and comparative thin-layer chromatography with standards, and the thin-layer chromatography of hydrolysates and hydrolysis products of dinitrophenyl derivatives. Valine derived from the peptide and diketopiperazine was shown to be of the L configuration by L-amino acid oxidase. L-[^{14}C]Valine and [^{3}H]glycine were incorporated into the glycine enzyme products in a 4:3 ratio; the deviation from the expected 1:1 ratio may be due to the loss of tritium from [^{3}H]glycine bound as the thioester since hydrogens in α-position to thioesters can exchange with water (Bruice and Benkovic, 1966).

The formation of both valylglycine and the corresponding diketopiperazine were observed to be ATP dependent. The ratio of dipeptide to diketopiperazine formed was a function of the pH of the incubation mixture: 4 or 5:1 at pH 7.3–7.4; 1.5:1 at pH 7.8–7.9. Normally, 5–6 mol of free dipeptide + diketopiperazine were found per mol of glycine enzyme after a 30-min incubation, and compared with the enzyme-bound dipeptide, 20–50 times as much dipeptide was free, presumably released into solution.

Experiments with Purified Alanine Enzyme. Table IV lists the amino acid dependency of the ATP-PP_i exchange catalyzed by the alanine enzyme. The enzyme reacted well with the L forms of valine and leucine but showed only negligible exchange with their D forms; these appear to be formed by racemization after thioesterification of the L forms. Sections 2 and 3 of Table IV show lack of ATP-PP_i exchange dependent on glycine or on the amino acids in tyrocidine, ornithine and proline, including phenylalanine and tryptophan which also appear in the later portion of linear gramicidin. This indicates the absence of the corresponding activating enzymes in this preparation.

The binding of amino acids in thioester linkage is presented for the alanine enzymes in Table IV. This binding increased with increasing amino acid concentrations, while the ratio of bound leucine to alanine to valine was nearly 1:2:2 over the concentration range examined. As with the glycine enzyme, the less than stoichiometric binding of labeled amino acid to enzyme is analogous to the similar finding with the tyrocidine enzymes (Lee et al., 1973).

The alanine enzyme was found to be unstable, readily dissociating (Figures 2 and 5) into forms that correspond in position to the expected molecular weight of polyenzymes composed of two or three amino acid activating subunits of somewhat over 2 × 70 000 and 3 × 70 000, respectively. Many cell extracts, such as those prepared after the peak of β-alanine uptake or those stored for any length of time prior to gel filtration, contained L-alanine-dependent ATP-PP_i exchange activities only in the 160 000 molecular weight region of the Sephadex G-200 gel filtration profiles, corresponding to a two amino acid activating subunit.

Formation of Peptides. We mentioned above evidence for the existence of some L-valylglycine bound as the thioester to the glycine enzyme. Table VI presents the results obtained when both glycine and alanine enzymes were incubated with ATP, Mg^{2+}, and various combinations of labeled and unla-

TABLE VI: Formation of Protein-Bound Nascent Peptide Chains.[a]

Expt	Amino acid L-Val	Gly	L-Ala	L-Leu	Moles of bound amino acid/mol of Ala enzyme
1	^{14}C	+	-	-	0.36
2	^{14}C	+	+	-	0.62
3	^{14}C	+	+	+	2.27
4	+	^{14}C	-	-	0.07
5	+	^{14}C	+	-	0.91
6	+	^{14}C	+	+	1.56
7	-	-	^{14}C	+	0.17
8	+	-	^{14}C	-	0.18
9	+	+	^{14}C	-	0.66
10	+	+	^{14}C	+	2.90

[a] Protein-bound nascent peptides were assayed as described (Roskoski et al., 1970a). Labeled amino acids (1 μCi) were 0.1 mM and designated ^{14}C in the table. Unlabeled (L configuration) amino acids were 1.0 mM and are designated +; a dash (-) indicates deletion of that amino acid. Each sample (final pH 7.6) contained 100 μg of glycine enzyme (from hydroxylapatite) and 330 μg of alanine enzyme (from DEAE-cellulose 2).

beled amino acids. These results are more complex than those obtained with the tyrocidine synthesizing system (Roskoski et al., 1970b); however, the following conclusions can be made. To obtain the maximum incorporation of label into a trichloroacetic acid precipitable form, all four amino acids present in the initiating heptapeptide of linear gramicidin must be added. The exclusion of one of them strongly reduces the binding of labeled amino acid to the trichloroacetic acid precipitated enzymes. This indicates that here also the enzyme-bound peptides of specific sequence are formed by one-by-one addition and that extension on the alanine enzyme requires peptidyl transfer from the glycine enzyme.

Discussion

We anticipated that the easiest progress toward a better understanding of linear gramicidin synthesis could be made by concentrating on those enzymes that activate the two amino acids, glycine and alanine, present exclusively in this antibiotic, glycine being the second and alanine the third and fifth amino acids in its sequence. It could be concluded already from its position in the crude extract after analysis on Sephadex G-200 and by comparing its position with those of the tyrocidine enzymes that glycine was activated on a small enzyme, while the position of the main alanine peak indicated it was activated by a larger enzyme.

Purified preparations of the two enzymes indicated molecular weights of 150 000 and 350 000, respectively, for the two enzymes. Following the example of the tyrocidine enzymes, division by the number of amino acids activated yields an approximate molecular weight of 70 000 for every amino acid activating subunit. Therefore, the glycine enzyme should activate two amino acids and the alanine enzyme five amino acids. This conclusion is also supported by the nearly 1:1 binding of valine and glycine to the glycine enzyme and the nearly 1:2:2 binding of leucine, alanine, and valine to the alanine enzyme. The results reported indicate that with a mixture of the glycine and alanine enzymes, omission of an amino acid interrupts the sequence of seven synthesized by the two enzymes, indicating that the amino acids are added one by one in sequence.

In this study, no further attempts were made to characterize the enzymatic nature of the synthesis of linear gramicidin beyond the heptapeptide stage. Preliminary, unpublished experiments by one of us (S.G.L.) indicate the final sequence may be synthesized by enzymes that activate two amino acids each.

The initiation of an in vitro synthesis of linear gramicidin appears to be a function of the glycine enzyme which activates the valine-glycine sequence. A complication in this interpretation arises from the fact that the first amino acid in the in vivo synthesized antibiotic is a formylvaline that is neither activated nor incorporated into the isolated enzyme system. Bauer et al. (1972) found, however, that the inhibition of transformylation by aminopterin inhibits the synthesis of linear gramicidin and markedly increases the formation of tyrocidine.

Some features of the glycine enzyme deserve attention since, to some extent, they are unusual. When offered both valine and glycine, purified glycine enzyme retains only a small amount of enzyme-bound dipeptide and releases a rather large amount of dipeptide into the supernatant fraction together with a smaller quantity of L-Val-Gly-diketopiperazine.

While this work was in progress, a paper by Akashi et al. (1977) appeared in which they report on the isolation of a fraction from *B. brevis* (ATCC 8185) that is comparable to our glycine enzyme. With this fraction, fortified by the addition of a crude *B. brevis* fraction and a pigeon liver formyltransferase, they obtained formylation of enzyme-bound valine and valine-glycine. This appears to indicate a stabilization of enzyme-bound valine-glycine by formylation because, as mentioned, we observed an unusually large release of enzyme-bound valine-glycine. The free valyl-L-glycine dipeptide does not cause an ATP-PP_i exchange, nor is it incorporated when offered to the glycine enzyme, confirming the observation by Kurahashi's group (Kurahashi, 1961; Tomino and Kurahashi, 1964) with gramicidin S synthesis that released peptides are not reincorporated into the antibiotic.

In conclusion, linear gramicidin is synthesized by the mechanism found for tyrocidine and also for the synthesis of a number of other polypeptides studied in other laboratories, as indicated by the presence of 4′-phosphopantetheine in the two enzymes that initiate the synthesis of this antibiotic and as preliminarily shown by the work of Bauer et al. (1972). So far, however, all examples of thioester-linked synthesis have been found in prokaryotes, none as yet in a eukaryote cell (Bauer and Lipmann, 1976).

References

Akashi, K., Kubota, K., and Kurahashi, K. (1977), *J. Biochem. (Tokyo) 81,* 269.

Akers, H., Lee, S. G., and Lipmann, F. (1976), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35,* 1533.

Bauer, K., and Lipmann, F. (1976), *Endocrinology 99,* 230.

Bauer, K., Roskoski, R., Jr., Kleinkauf, H., and Lipmann, F. (1972), *Biochemistry 11,* 3266.

Beers, R. F., Jr., and Sizer, I. W. (1952), *J. Biol. Chem. 195,* 133.

Bruice, T. C., and Benkovic, S. (1966), Bioorganic Mechanisms, Vol. 1, New York, N.Y., W. A. Benjamin, p 268.

Frøshov, Ø., Zimmer, T. L., and Laland, S. G. (1970), *FEBS Lett. 7,* 68.

Gevers, W., Kleinkauf, H., and Lipmann, F. (1969), *Proc. Natl. Acad. Sci. U.S.A., 63,* 1335.

Hotchkiss, R. D. (1944), *Adv. Enzymol. 4,* 153.

Kiseler, N. A., Shpitzberg, C. L., and Vainshtein, B. K. (1967), *J. Mol. Biol. 25,* 433.

Kubota, K., and Kagawa, K. (1976), in *Sc. Rep. Himeji Inst.*

Technol., No. 29, Ser. A, p 31.
Kurahashi, K. (1961), Abstract, 5th International Congress of Biochemistry, Moscow, Pergamon Press, Oxford, p 37.
Lee, S. G., and Lipmann, F. (1975), *Methods Enzymol. 43,* 585.
Lee, S. G., Littau, V., and Lipmann, F. (1975), *J. Cell Biol. 66,* 233.
Lee, S. G., Roskoski, R., Jr., Bauer, K., and Lipmann, F. (1973), *Biochemistry 12,* 398.
Lipmann, F. (1973), *Acc. Chem. Res. 6,* 361.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 196,* 265.
Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236,* 1372.
Mazer, R. H., Ellis, B. W., and Cammarata, P. S. (1962), *J. Biol. Chem. 237,* 1619.
Porter, R. R. (1957), *Methods Enzymol. 4,* 221.
Pugh, E. L., and Wakil, S. J. (1965), *J. Biol. Chem. 240,* 4727.
Roskoski, R., Jr., Gevers, W., Kleinkauf, H., and Lipmann, F. (1970a), *Biochemistry 9,* 4839.
Roskoski, R., Jr., Kleinkauf, H., Gevers, W., and Lipmann, F. (1970b), *Biochemistry 9,* 4846.
Sarges, R., and Witkop, B. (1965), *J. Am. Chem. Soc. 87,* 2011.
Tomino, S., and Kurahashi, K. (1964), *Biochem. Biophys. Res. Commun. 17,* 288.
Zamenhof, S. (1957), *Methods Enzymol. 3,* 696.

Effect of Cations on tRNA Structure†

P. H. Bolton and D. R. Kearns*

ABSTRACT: The structure of tRNA in the presence of certain cations has been investigated by high-resolution nuclear magnetic resonance (NMR). In the presence of 0.17 M Na at 22 °C the structure of tRNA is similar to, but not identical with, the native structure. At higher temperatures the difference between the native structure and the structure in the presence of 0.17 M Na increases. Magnesium sequentially stabilizes the tRNA structure in the presence of 0.17 M Na in the temperature range 22–44 °C, the number of magnesium required for the stabilization of the native structure increases from 2 ± 1 at 22 °C to 4 ± 1 at 44 °C, and the number of interactions with slowly exchanging hydrogen-bonded imino protons stabilized increases from 1 ± 1 at 22 °C to 4 ± 1 at 44 °C. The polyamines spermine and spermidine stabilize some, but not all, of the interactions stabilized by magnesium. For tRNA in the presence of 0.17 M Na at 44 °C, 3–4 spermine per tRNA stabilizes 2 ± 1 interactions with slowly exchanging hydrogen-bonded imino protons. The combination of spermine and magnesium was found to be more effective than either cation alone in stabilizing the native structure of tRNA. The NMR results offer no evidence that the structure of tRNA in the presence of both spermine and magnesium (at high levels of both cations) is different from the native structure. In the presence of tetraethylammonium or tetramethylammonium ions, the tRNA structure is extensively destabilized relative to the native structure. Addition of magnesium to such samples stabilizes the native structure in a nonsequential manner, and about eight magnesium per tRNA are needed for the stabilization. The NMR results are taken in conjunction with the results of other investigators to propose a comprehensive model for the interaction of tRNA with cations. The use of assignments of some resonances to specific tertiary interactions suggests that magnesium stabilizes the tertiary interactions of tRNA in the following order: $s^4U_8 \cdot A_{14}$, U_{33}, $A_{58} \cdot T_{54}$, and $G_{19} \cdot C_{56}$.

There has been considerable interest in determining the effect of various cations on the structure and stability of tRNA (Römer and Hach, 1975). Cations associated with tRNA in vivo are of special interest. A number of different experimental techniques have been used to investigate the interaction of polyvalent cations with tRNA and other polynucleotides (Römer and Hach, 1975; Sander and Ts'o, 1971; Danchin, 1972; Stein and Crothers, 1976a,b; Schrier and Schimmel, 1974, 1975; Pochon and Cohen, 1972; Kayne and Cohn, 1974; Wolfson and Kearns, 1975; Jones and Kearns, 1974; Lynch and Schimmel, 1974; Bina-Stein and Stein, 1976).

Studies of tRNA interaction with cations can be separated into two broad groups: those primarily concerned with the metal binding properties of tRNA (i.e., the number of binding sites, equilibrium constants) and those which have focused on the effect of cations on tRNA structure. It has been shown that tRNAs have about five strong binding sites for magnesium, with binding constants of about 10^5 M^{-1} in a wide variety of experimental conditions (Römer and Hach, 1975; Danchin, 1972; Schrier and Schimmel, 1974, 1975; Stein and Crothers, 1976a,b; Bina-Stein and Stein, 1976). From such studies it is known that tRNAs exhibit a set of strong polyvalent cation binding sites not found in either tRNA fragments or double helical or single-stranded RNA. Furthermore, there is considerable evidence that the strong binding sites are intimately associated with tRNA tertiary structure (Römer and Hach, 1975; Kayne and Cohn, 1974; Stein and Crothers, 1976b; Bina-Stein and Stein, 1976; Bolton and Kearns, 1977). However, recent reports have shown that in the presence of high levels of sodium, 0.17 M, tRNAs have only one or two strong binding sites at 4–22 °C (Stein and Crothers, 1976a,b; Bina-Stein, 1976; Bolton and Kearns, 1977). The manner in which magnesium and spermine bind to tRNA (cooperative, independent, or sequential) also appears to depend on the experi-

† From the Department of Chemistry, Revelle College, University of California, San Diego, La Jolla, California 92093. *Received December 14, 1976; revised manuscript received August 30, 1977.* This work was supported by a grant from the U.S. Public Health Service (GM 22969).