Affinity Labeling of the Virginiamycin S Binding Site on Bacterial Ribosome†

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ABSTRACT: Virginiamycin S (VS, a type B synergimycin) inhibits peptide bond synthesis in vitro and in vivo. The attachment of virginiamycin S to the large ribosomal subunit (50S) is competitively inhibited by erythromycin (Ery, a macrolide) and enhanced by virginiamycin M (VM, a type A synergimycin). We have previously shown, by fluorescence energy transfer measurements, that virginiamycin S binds at the base of the central protuberance of 50S, the putative location of peptidyltransferase domain [Di Giambattista et al. (1986) *Biochemistry 25*, 3540–3547]. In the present work, the ribosomal protein components at the virginiamycin S binding site were affinity labeled by the *N*-hydroxysuccinimide ester derivative (HSE) of this antibiotic. Evidence has been provided for (a) the association constant of HSE–ribosome complex formation being similar to that of native virginiamycin S, (b) HSE binding to ribosomes being antagonized by erythromycin and enhanced by virginiamycin M, and (c) a specific linkage of HSE with a single region of 50S, with virtually no fixation to 30S. After dissociation of covalent ribosome–HSE complexes, the resulting ribosomal proteins have been fractionated by electrophoresis and blotted to nitrocellulose, and the HSE-binding proteins have been detected by an immunoenzymometric procedure. More than 80% of label was present within a double spot corresponding to proteins L18 and L22, whose R_fs were modified by the affinity-labeling reagent. It is concluded that these proteins are components of the peptidyltransferase domain of bacterial ribosomes, for which a topographical model, including the available literature data, is proposed.

Naturally occurring virginiamycin-like antibiotics (synergimycins) are mixtures of two kinds of chemically unrelated molecules (types A and B) endowed with synergistic inhibitory activity on microorganisms (Vãzquez, 1975; Tanaka, 1975; Cocito, 1979). The minimal growth inhibitory concentration of type B components is lowered hundredfold by type A components. Virginiamycin S (VS,[1] type B synergimycin) inhibits peptide bond synthesis and causes the release of incomplete polypeptide chains, in vitro and in vivo (Chinali et al., 1988a,b; Di Giambattista et al., 1989). Its molecular mechanism of action is similar to that of erythromycin (Ery, a 14-membered macrolide). Virginiamycin M (VM, a type A synergimycin) promotes the release of aminoacyl-tRNA from the ribosomal A site and the translocational ejection of peptidyl-tRNA from the P site (Chinali et al., 1981, 1984; Cocito & Chinali, 1985). Both A and B synergimycins bind to 50S ribosomal subunits with 1:1 stoichiometry. Virginiamycin S binding can be quantitatively measured spectrofluorometrically (Parfait et al., 1978). By this technique, the kinetic constants for the ribosome-binding reactions of virginiamycin S and related antibiotics have been determined (Moureau et al., 1983; Di Giambattista et al., 1987).

Affinity-labeling techniques have been widely used to map different ribosomal components. Moreover, analysis of chromosomal resistance to antibiotics has pointed to mutational changes of either L proteins or 23S rRNA. This is exemplified by modifications of L4 and L22 leading to erythromycin resistance (Wittmann et al., 1973) and by the A 2058 dimethylation conferring "undissociated" resistance to the related families of macrolides, lincosamides, and type B synergimycins (MLS) (Weisblum & Demohn, 1969; Weisblum et al., 1971). Also, an investigation on the protection of 23S rRNA against nucleic acid reagents has identified some rRNA segments specifically shielded by antibiotics (Moazed & Noller, 1987). These results matched those of sequencing of in vivo and in vitro mutagenized 23S rRNA gene (Sigmund & Morgan, 1982; Sigmund et al., 1984; Ettayebi et al., 1985; Vester & Garrett, 1987).

The location of the virginiamycin S binding site on bacterial ribosomes has been established by nonradiant energy transfer between ribosome-bound virginiamycin S (endowed with inherent fluorescence) and fluorescent coumarinyl residues grafted onto L7, L10, and L12 proteins of known topological situation. By this work, a region at the base of central protuberance, the putative peptidyltransferase domain, has been identified (Di Giambattista et al., 1986). This observation, in addition to the virginiamycin S promoted inhibition of peptide bond formation (Chinali et al., 1988a,b), has suggested the possibility of labeling the peptidyltransferase domain by use of an affinity-labeling derivative of virginiamycin S. The main difficulties for this kind of approach are as follows: (a) chemically produced alterations often inactive antibiotics; (b) most synthesized derivatives are unstable; (c) grafted reactive arms frequently undergo aspecific covalent bond formation; (d) dissociation of ribosomal complexes for identification of labeled components may produce a loss of label. We have recently synthesized active and stable virginiamycin S derivatives, which have been used in the present work to elicit the synthesis of anti-virginiamycin S antibodies and to affinity label the virginiamycin S binding site within the peptidyltransferase center. The simplified structure of the antibiotic is shown in Figure 1.

Materials and Methods

Chemicals. Carboxymethoxylamine hemihydroxychloride, diphenylphosphoryl chloride (DPPC), 1-ethyl-3-[3-(di-

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[1] Abbreviations: VM, virginiamycin M, a type A synergimycin; VS, virginiamycin S, a type B synergimycin; MLS, a group of antibiotics including the macrolides, lincosamides, type B streptogramins (synergimycins) families; HSE, *N*-hydroxysuccinimide ester of virginiamycin S.

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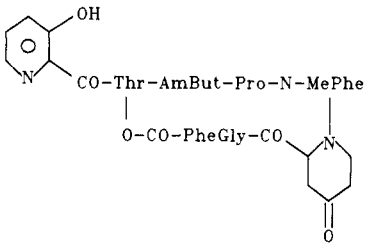

FIGURE 1: Structure of virginiamycin S1.

methylamino)propyl]carbodiimide hydrochloride (EDC), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), *p*-nitrotetrazolium blue (NTB), and *N*-hydroxysuccinimide (HSI) were from Sigma Chemical Co. (St. Louis, MO) and India ink (Pelikan Fount India) was from Pelikan AG (Hannover, Germany). Only reagent grade solvents were used.

Buffers. The following buffers were used in different experiments: TEA (50 mM triethanolamine, pH 7.8, 10 mM $MgCl_2$, 100 mM KCl, and 4% EtOH); TMA (20 mM Tris-HCl, pH 7.4, 80 mM NH_4Cl, and 10 mM $MgCl_2$); TBS (50 mM Tris-HCl, pH 7.4, and 200 mM NaCl); TMS (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$); TSA (0.02 M Tris-HCl, pH 8, 0.028 M NaCl, and 0.02% NaN_3); TSE (0.05 M Tris-HCl, pH 7.5, 0.1 M NaCl, and 0.1 mM EDTA); TGM (0.025 M Tris-HCl, pH 8.3, 0.195 M glycine, and 20% methanol); BMA (0.02 M borate, pH 8, 10 mM $MgCl_2$, and 100 mM NH_4Cl).

Biologicals. Bovine serum albumin, lactalbumin, and molecular weight markers were from Sigma. Aminoalkylagarose (Affi-Gel 102) was from Bio-Rad Laboratories (Richmond, CA). Sephadex, Sepharose 4B, and protein A–Sepharose CL-4B were from Pharmacia (Uppsala, Sweden). Anti-rabbit IgG alkaline phosphatase conjugate was a product of Promega Corp. (Madison, WI).

Antibiotics. Virginiamycin M and S were crystallized from the fermentation medium of *Streptomyces virginiae* (SKF-RIT Laboratories, Rixensart, Belgium). Erythromycin was from Boehringer (Mannheim, Germany).

Preparation of Ribosomes and Components. Ribosomes and subunits were prepared from *Escherichia coli* A19 as previously described (Di Giambattista et al., 1987). Ribosomal proteins were extracted either by urea–LiCl (Spitnik-Elson, 1965) or by acetic acid (Hardy et al., 1969).

Fluorescence Measurements. Binding of virginiamycin S and derivatives to *E. coli* ribosomes was measured spectrofluorometrically, the enhancement of intrinsic fluorescence of this antibiotic in the presence of ribosomes being proportional to ribosome–virginiamycin S concentration. The excitation monochromator was set at 330 nm, and the fluorescence emission was recorded at 420 nm (SPR-Ratio II Aminco-Bowman spectrofluorometer at 25 °C) (Di Giambattista et al., 1987). Concentration of bound antibiotic (VS_B) was determined from total virginiamycin S in solution according to the equation

$$(VS_B) = \frac{F - F_F}{F_F(\gamma - 1)}(VS_T)$$

where F and F_F were the fluorescence intensities, in the presence and absence of ribosomes, respectively, and γ was a constant representing the ratio of fluorescence per mole of bound virginiamycin S to that of free virginiamycin S. The γ value was determined by adding a large excess of ribosomes to virginiamycin S solutions of known concentration.

Synthesis of Virginiamycin S Derivatives for Affinity Labeling. (1) Oximation of virginiamycin S (VS–COOH) was carried out as follows. A solution of 0.02 mmol of virginiamycin S and 0.06 mmol of carboxymethoxylamine in 1 mL of EtOH containing 5 μL of pyridine was stirred for 2 h at room temperature. Reaction was monitored by thin-layer chromatography on silica plates (Sil G 0.2 mm thick plates from Merck, Darmstadt, FRG) with the solvent chloroform/acetic acid/methanol (92:4:4 v/v) (the R_f of virginiamycin S was 0.62 and that of the oximated product 0.32). Water (300 μL) and concentrated HCl (10 μL) were added, and the mixture was extracted with chloroform (2 mL). The organic phase was repeatedly washed with water and evaporated to dryness, after being mixed with 2 mL benzene. Two isomers were obtained (90% yield), with R_f of 0.13 and 0.10 [thin-layer chromatography on silica plates with the chloroform/acetic acid mixture (95:5)]. Infrared spectrum showed a lactone band at 1739 cm^{-1} (overlapping with acid CO) and an oxime band at 1675 cm^{-1}. The ^1H NMR ($CDCl_3$) analysis confirmed the presence of two isomers (54/46 ratio) with spectra similar to that of the native compound, except for an additional AB spin system [$NOCH_2COOH$ at δ 4.56 (major) and δ 4.23 (minor) (Anteunis et al., 1988)].

(2) *N*-Hydroxysuccinimido diphenyl phosphate (SDPP) was prepared according to the following protocol (Ogura et al., 1980). To 0.01 mol of diphenylphosphoryl chloride (DPPC) in 6 mL of dichloromethane were added 0.01 mol of triethylamine and 0.01 mol of *N*-hydroxysuccinimide dropwise (0 °C, 35 min), and the mixture was dried in vacuo, repeatedly washed with ether, dissolved in ethyl acetate, and washed twice with 10 mL of water. By evaporation of the organic phase to dryness, ^1H NMR pure SDPP in 85% yield was obtained. The spectrum showed a peak at δ 2.89 (4 H, succinimide CH_2). The R_f of this product in thin-layer chromatography [silica gel plates and chloroform/acetic acid/methanol (92:4:4)] was 0.57, and the melting point was 89 °C.

(3) The *N*-hydroxysuccinimide ester of virginiamycin S–COOH (HSE) was synthesized from products **1** and **2**. To 100 μmol of virginiamycin S–COOH (section 1) in 1 mL of acetonitrile were added 150 μmol of triethylamine (or pyridine) and 150 μmol of SDPP (section 2) dissolved in 2 mL of acetonitrile (24 h, 20 °C, in dark). The mixture was centrifuged 15 min at 5000 rpm; the supernatant contained HSE in 60% yield, which was purified by preparative thin-layer chromatography [silica plates and chloroform/acetic acid/methanol (92:4:4)]. Purified HSE, which was further used for affinity labeling, had a R_f of 0.50. The ^1H NMR spectrum revealed the same characteristic of virginiamycin S–COOH plus a new peak at δ 2.86 (4 H, succinimide CH_2). The fluorescence properties (excitation wavelength at 345 nm and emission at 420 nm) of both virginiamycin S–COOH (**1**) and HSE (**3**) were similar to those of native virginiamycin S. The whole synthetic process described in sections 1 and 3 (patent pending) is illustrated in Figure 2.

(4) Coupling of HSE to bovine serum albumin (BSA–virginiamycin S) was obtained by the following protocol. To 1.4 μmol of *N*-hydroxysuccinimide ester of virginiamycin S in 100 μL of acetonitrile were added 2 μL of triethylamine, 350 μL of ethanol, 150 μL of 0.1 M borate buffer, pH 8.45, and 0.143 μmol of BSA dissolved in 100 μL of water (24 h, 20 °C, in dark). After addition of 50 μL of KCl (2 M) and 8 mL of ethanol, the mixture was stored at −20 °C for 4 h and centrifuged at 10000 rpm for 15 min. The precipitate was dried, resuspended in 1 mL of water and recentrifuged twice. The resulting product was loaded on a 10 mL Sephadex G-150 column and eluted with phosphate buffer (50 mM pH 7.8): the BSA–virginiamycin S containing fractions were pooled and

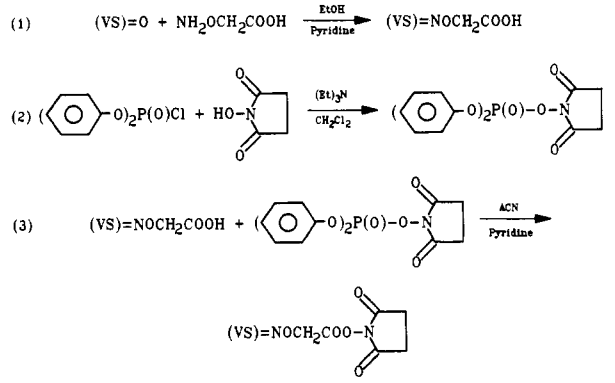

FIGURE 2: Synthetic pathway of the *N*-hydroxysuccinimide ester derivative of virginiamycin S1.

dialyzed against the same buffer plus 0.1 M NaCl. The virginiamycin S/BSA ratio was 15:1 (mole/mole) as determined by fluorescence measurements (excitation, 350 nm; emission, 420 nm).

(5) Virginiamycin S was coupled to agarose according to the following procedure. To 10 mL of Affi-Gel 102 (aminoalkylagarose containing approximately 150 μmol of amino groups) were added 33 μmol of virginiamycin S–COOH (**1**) in 5 mL of ethanol and 1 mL of water (adjusted to pH 4.5 with 0.1 N HCl), followed by 106 μmol of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) dissolved in 2 mL of water (14 h, 20 °C, dark). The unreacted ligand, carbodiimide, and urea derivative were washed away with 5 bed volumes of a 1:1 mixture of water/EtOH. The gel was equilibrated with TBS buffer containing 0.02% NaN_3 and stored at 4 °C.

Preparation of Virginiamycin S Specific IgG. (1) For preparation of polyclonal anti-virginiamycin S antiserum, rabbits were weekly injected with 0.5 mg of albumin–virginiamycin S conjugate (see above) in 0.5 mL of saline emulsified with an equal volume of complete Freund's adjuvant. Inoculation was repeated three times, and blood was collected 1 month later. Serum IgGs were purified by affinity chromatography on a 2.5-mL protein A–Sepharose CL-4B column (1 g corresponding to 3.5 mL of gel and containing 2 mg of protein A/mL) equilibrated with 5 bed volumes of TSA buffer. The serum, dialyzed against the same buffer, was applied to the column and eluted with TSA buffer until a negligible A_{280nm} value was obtained. IgGs were eluted with 50 mM glycine hydrochloride, pH 2.8, and dialyzed against TBS buffer. Virginiamycin S specific IgGs were obtained by a further chromatographic step on a virginiamycin S–agarose column (see section 5 under Synthesis of Virginiamycin S Derivatives for Affinity Labeling).

(2) Immunological testing of anti-virginiamycin S IgG was carried out on immunodiffusion plates, containing 1% agarose in TSE buffer. A sample of anti-virginiamycin S antiserum (15 μg) was placed in the center well, and increasing amounts (7, 11, and 22 μg) of BSA–virginiamycin S were placed in the peripheral wells. Diffusion was allowed to occur overnight at room temperature. Plates washed with 0.9% NaCl and air-dried were stained with Coomassie blue.

Affinity Labeling of Ribosomes with N-Hydroxysuccinimide Ester of Virginiamycin S (HSE). 70S ribosomes (5 nmol) from *E. coli*, previously reactivated 5 min at 45 °C in 1 mL of BMA buffer, were mixed with HSE (25 nmol) (see section 3 under Synthesis of Virginiamycin S Derivatives for Affinity Labeling) at 4 °C, leading to virginiamycin S–ribosome complex formation. The mixture was then incubated 30 min at 30 °C for covalent bond formation, and the reaction was stopped by the addition of ethanolamine. Further incubation (37 °C, 15 min) with 25 nmol of erythromycin (Ery) was allowed to displace noncovalently bound virginiamycin S (erythromycin competes with virginiamycin S for binding to ribosomes). Ribosome complexes were precipitated by addition of 700 μL of EtOH at −20 °C and 100 μL of 1 M $MgCl_2$, collected by centrifugation at 10000 rpm for 5 min, resuspended in 1 mL of TEA buffer, and recentrifuged twice. Percentage of labeled ribosomes was determined by fluorescence measurements of bound virginiamycin S (see above), assuming a correspondence of 1 A_{260nm} with 25 pmol of 70S. Nonspecific labeling was evaluated by saturating ribosomes with erythromycin (25 nmol) before the labeling step with the virginiamycin S ester.

Electrophoretic Fractionation of Ribosomal Proteins. (1) Ribosomal proteins were separated by one-dimensional or two-dimentional gel electrophoresis (Kyriakopoulos & Subramanian, 1976; Dijk & Littlechild, 1979; Subramanian, 1974; Mets & Bogorad, 1974; Kaltschmidt & Wittmann, 1970) on polyacrylamide gels, which were equilibrated 1 h in TGM buffer. Protein transfer to nitrocellulose sheets was carried out at 8 V/cm (16 h at 4 °C) (Towbin et al., 1979). Blots were washed 5 min with TBS buffer and heated at 80 °C for 45 min.

(2) Immunoblotting of affinity-labeled ribosomal proteins was performed as follows. Nitrocellulose sheets, in direct contact with the gel, were soaked 1 h in a 3% lactalbumin solution in TBS buffer, rinsed with this buffer, and incubated 1 h with anti-virginiamycin S antibodies (1/400 dilution of a 3 mg/mL IgG stock solution in 1% lactalbumin). The sheet was washed with TBS (five changes during 30 min) and incubated 1 h with alkaline phosphatase labeled anti-rabbit IgG (1/7500 dilution). After repeated washing with TBS–0.3% Tween 20 and a final TBS wash, *p*-nitrotetrazolium blue (0.63 mg/mL) and 5-bromo-4-chloro-3-indolyl phosphate (a phosphatase substrate, 0.16 mg/mL in TMS buffer) (Blake et al., 1984) were added. After 35 min, the reaction was stopped by washings with water, and the stained sheets were photographed and analyzed by a scanning densitometer.

Protein Staining. Reference electropherograms were obtained by transferring ribosomal proteins from gels to two overposed nitrocellulose sheets, one of which was submitted to direct staining (1 μL/mL of India ink in washing buffer) (Hancock & Tsang, 1983) and the other to the immunoblotting technique. In some cases, blots were stained after immunorevelation.

Densitometric Measurements. Nitrocellulose sheets submitted to the immunoenzymometric procedure were scanned in the reflective mode by a densitometer (Chromoscan 3, Joyce-Loebel, Gateshead, U.K.). The corresponding parameters were excitation wavelength 530 nm, aperture width 0.3 mm, and absorbance maximum 0.5 OD unit. Background corrections were made by the valley-to-valley procedure (calculation of straight line fits between adjacent troughs).

RESULTS

Analysis of Virginiamycin S Derivatives. Biological activity of affinity-labeling derivatives must be comparable to that of the corresponding natural compounds (a prerequisite for specific interaction with the target site). Moreover, their binding to ribosomes should occur under conditions preventing the establishment of a covalent bond. Experiments were performed to prove that the *N*-hydroxysuccinimide ester derivative of virginiamycin S (HSE), which was synthesized according to a procedure outlined under Materials and Methods, did indeed fulfill these two requirements.

Table I: Comparative Biochemical Properties of Virginiamycin S and Its Derivatives

antibiotics[a]	chromatographic migration[b] (R_f)	mol wt	^{1}H NMR spectrum[c] (δ)	ribosome binding[d] ($K_a \times 10^6$ M^{-1})
virginiamycin S (VS)	0.62	825		3.2
oxime derivative of VS (VS-COOH)	0.32[e]	898	4.56 (4.23) ($NOCH_2COOH$)	2.1
succinimide ester of VS (HSE)	0.50	995	2.86 (4 H, succinimide CH_2)	1.7

[a] Preparation and analytical procedures outlined under Materials and Methods. [b] Thin-layer chromatography on silica gel plates with chloroform/acetic acid/methanol (92:4:4). [c] Only the extra peaks not appearing in the VS spectrum are reported. [d] Association constants calculated from the binding curves displayed in Figure 1. [e] The two isomers produced in the oximation reaction are separable (R_f 0.13 and 0.10) by thin-layer chromatography [silica gel plates and chloroform/acetic acid (95:5)].

Table II: Ribosome-Bound HSE Derivative[a] of Virginiamycin S under Different Experimental Conditions

sample[b]	antibiotics[a,c] step 1	step 2	step 3	ribosome-bound HSE[d] (mol of HSE/mol or ribosome)
1	none	HSE	Ery	0.35–0.42
2	VM	HSE	Ery	0.51–0.65
3	Ery	HSE	Ery	0.05–0.08
4	none	HSE*	Ery	undetectable

[a] Abbreviations: VS and VM, virginiamycins S and M; Ery, erythromycin; HSE, succinimide derivative of VS. [b] Reaction mixture: reactivated *E. coli* ribosomes (5 μM) in buffer TEA. [c] Step 1, preincubation (4 °C, 5 min) with different antibiotics (25 μM). Step 2, covalent reaction of the HSE derivative of VS (30 °C, 30 min), except in sample 4 (blank of hydrolyzed HSE*). Step 3, incubation with excess of erythromycin (25 μM) to remove noncovalently bound HSE. [d] Spectrofluorometric measurement of ribosome-bound VS derivative (cf. Materials and Methods).

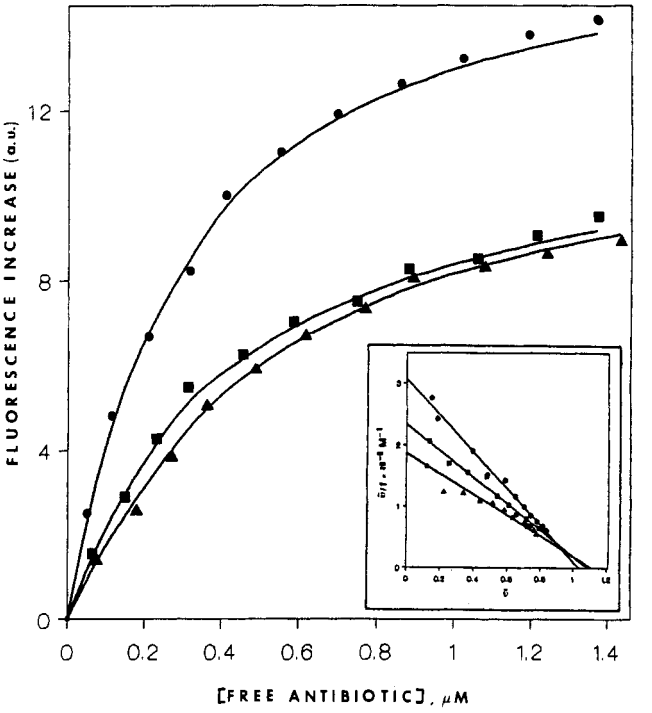

FIGURE 3: Binding of virginiamycin S and its derivatives to *E. coli* ribosomes. Suspensions of 70S ribosomes (1 μM, TEA buffer, 4 °C) were mixed with increasing concentrations (0.2–2.2 μM) of either virginiamycin S (●), its oxime derivative (■), or the *N*-hydroxysuccinimide ester derivative (▲). Fluorescence intensity (excitation, 330 nm; emission, 420 nm; 10-nm slits) was recorded 30 s after the addition of antibiotics. Concentration of bound antibiotic was determined as described under Materials and Methods. Data were analyzed according to the procedure of Scatchard (1949). Values of bound VS/mole of ribosomes ($\bar{\nu}$) were plotted against $\bar{\nu}$/concentrations of free VS (f) (insert).

The binding reaction of native virginiamycin S has been compared to that of two derivatives, the oxime (virginiamycin S–COOH) and the hydroxysuccinimide ester (HSE). To a fixed concentration of ribosomes, increasing amounts of antibiotics were added, and the increase of fluorescence intensity was recorded. Similar ribosome–antibiotic binding curves were obtained in all cases (Figure 3). From the spectrofluorometric data, the concentration of bound antibiotic was calculated and the corresponding association constants were estimated according to the method of Scatchard (1949). Data in Table I indicate K_a values of 3.2×10^6, 2.1×10^6, 1.7×10^6 M^{-1} for the virginiamycin S, the oxime, and the *N*-hydroxysuccinimide ester, respectively. The stoichiometry of the binding reaction was equal to unity in all cases.

In step 1 of the experiments summarized in Table II, ribosomes were preincubated either with buffer (reference) or with a competing antibiotic (erythromycin) or with a synergistic antibiotic (virginiamycin M). In step 2, all samples (except control sample 4) were treated with HSE, under conditions allowing the establishment of a covalent bond. Noncovalently bound HSE was displaced from ribosomes by an excess of erythromycin (step 3), and ribosome-bound HSE was measured spectrofluorometrically. After pretreatment with erythromycin (sample 3), the affinity-labeling level was lowered to $^1/_7$ of the reference value of 0.35–0.42 mol of HSE/mol of ribosomes. Moreover, a preincubation of ribosomes with virginiamycin M (entailing a raise of ribosome affinity for virginiamycin S) induced a reproducible increase of the affinity-labeling level (sample 2). These data warranted the specificity of the binding reaction of HSE to ribosomes and suggested this virginiamycin S derivative would be a suitable reagent for affinity labeling the antibiotic binding site.

Immunological Analysis of an Antibiotic–Albumin Conjugate. The approach chosen for affinity labeling of the virginiamycin S binding site has been an immunoenzymometric procedure for identification of the antibiotic derivative covalently linked to ribosomal proteins. Anti-virginiamycin S antiserum was an essential reagent for this study; however, antibiotics are haptens unable to elicit per se antibody formation. This difficulty was circumvented by coupling the oxime derivative of the antibiotic to bovine serum albumin according to a procedure outlined under Materials and Methods. The corresponding BSA–virginiamycin S conjugate was injected to rabbit, and total immunoglobulins were separated from the resulting antiserum. Virginiamycin S specific IgGs were further purified by chromatography on an affinity column containing an agarose–virginiamycin S conjugate.

The experiment illustrated in Figure 4 was performed to check the immunoreactivity and specificity of the anti-virginiamycin S IgG. Different concentrations of virginiamycin S–albumin conjugate were allowed to react with the anti-virginiamycin S IgG in an immunodiffusion plate, and antigen–antibody complexes were stained. A specific reaction occurring with the conjugate, but not with albumin alone, was evident.

Affinity Labeling of 70S Ribosomes by a Virginiamycin S Derivative. The reaction of the HSE derivatives of this antibiotic with 70S ribosomes was explored under a variety of experimental conditions. An optimum yield of covalently linked HSE (0.35–0.42 mol of HSE/mol of ribosomes) was obtained with an input HSE/ribosome ratio of 5 and a 30-min incubation at 30 °C. The experimental conditions outlined

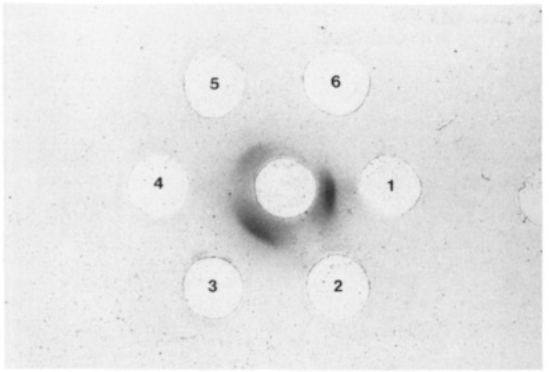

FIGURE 4: Analysis of the specificity of anti-virginiamycin S immunoglobulins. A sample (15 μg) of purified anti-virginiamycin S antibodies was placed in the center well, decreasing amounts of bovine serum albumin–virginiamycin S conjugate were placed in the peripheral wells (22, 11, and 7 μg of the conjugate in wells 1, 3 and 5, respectively), and a control of bovine serum albumin (11 μg) was placed in well 6. After immunodiffusion (14 h, 20 °C), plates were stained with Coomassie blue.

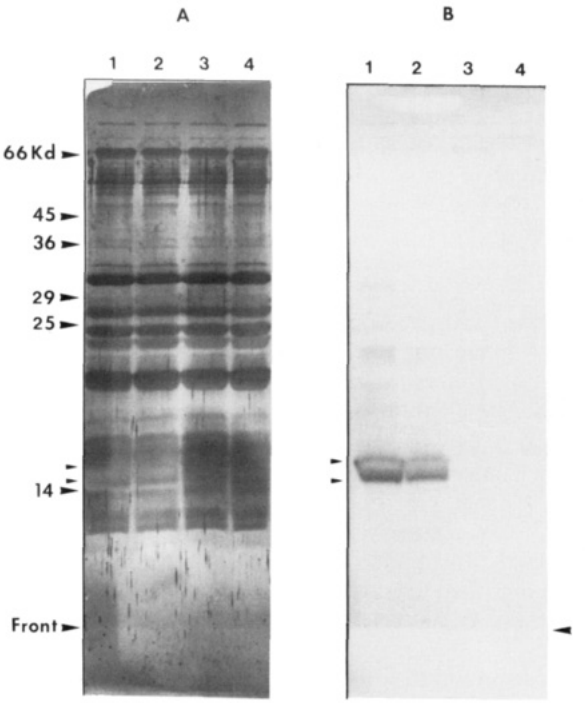

FIGURE 5: Affinity labeling of ribosomal proteins by a virginiamycin S derivative. Total 70S ribosomal proteins (120 μg) from covalent virginiamycin S–ribosomes complexes (samples I–IV of Table II) were fractionated by one-dimensional gel electrophoresis SDS–PAGE (Stacking gel: 4% acrylamide; 0.066% bis(acrylamide); 0.1% SDS; pH 6.8. Resolving gel: 15% acrylamide; 0.4% bis(acrylamide); 0.1% SDS; pH 8.8; 35 mA/gel; 5 h.) and electrophoretically transferred to nitrocellulose sheets (70 mA, 16 h). (Panel A) Pattern of ribosomal proteins after blot, staining with India ink (1 μl/mL in TBS buffer containing 0.1% Tween 20). (Panel B) Blots incubated with anti-virginiamycin S rabbit IgG and stained with alkaline phosphatase labeled anti-rabbit IgG antibodies. Samples: The labeling step with the HSE derivative of virginiamycin S was performed in the presence of virginiamycin M (lane 2), erythromycin (lane 3), or none (reference, lane 1). Blank of hydrolyzed HSE ester is in lane 4. Experimental conditions were as in Table II. Arrows indicate the position of standard proteins of known molecular weight.

in Table II (sample 1) were maintained in all subsequent experiments.

Ribosomes carrying covalently linked virginiamycin S (Table II) were dissociated, and the resulting ribosomal proteins, after fractionation by monodimensional polyacrylamide gel electrophoresis (SDS–PAGE), were transblotted to nitrocellulose

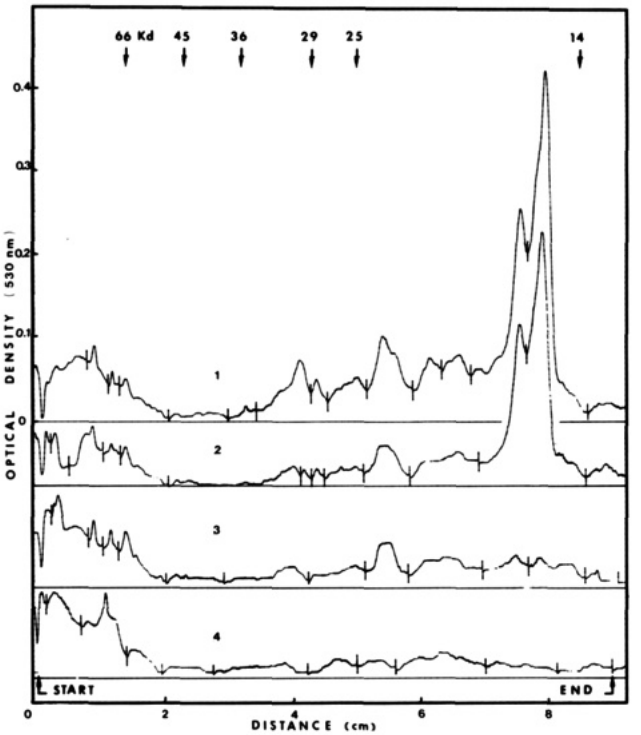

FIGURE 6: Densitometer tracing of affinity-labeled ribosomal proteins. Electropherograms shown in panel B of Figure 5 were analyzed by reflectance densitometry (λ_{350nm}; aperture width 0.3 mm, and absorbance maximum 0.5 OD). Arrows indicate the position of standard proteins of known molecular weight.

sheets. The protein-bound antibiotic was detected by an immunoenzymometric procedure with anti-virginiamycin S IgG. As shown in Figure 5, two major bands were labeled in samples 1 (reference) and 2 (preincubation of ribosomes with virginiamycin M before the affinity-labeling steps). On the contrary, no label was found when HSE binding to ribosomes was competitively inhibited by erythromycin (lane 3). The densitometric pattern of the immunoblot showed that 60–80% of total integrated areas were associated with two major bands of about 15 kDa (samples 1 and 2 in Figure 6). These results indicate the occurrence of a restricted number of targets, possibly two components (or groups of components), within the HSE–ribosome covalent complexes.

Localization of Labeled Sites within the Ribosomal Subunits. The HSE–70S ribosomes analyzed in the previous section were dissociated into the 50S and 30S subunits. Immunoblot analysis of subunit proteins fractionated by monodimensional electrophoresis is shown in Figure 7. Two major labeled bands were present in the large ribosomal subunit (samples 1 and 2 of panel B), whereas no reaction was recorded with S proteins from 30S (panel D). This result indicates a specific affinity labeling of L proteins by HSE.

Identification of Labeled Ribosomal Proteins. To identify the labeled ribosomal proteins, covalent HSE–70S complexes were fully dissociated, and proteins were fractionated by the bidimensional electrophoresis procedure of Mets and Bogorad (1974). With this method, proteins were separated according to their charges in the first dimension and according to their molecular weight in the second dimension. Proteins were transblotted to a couple of nitrocellulose sheets: one for immunodetection of labeled proteins and the other for all protein staining (reference). The double spot in Figure 8B correspond to a crowded region containing the closely located proteins L18, L19, and L22 (Figure 8A,C). To circumvent this difficulty, further fractionation was carried out with the Kaltschmidt and

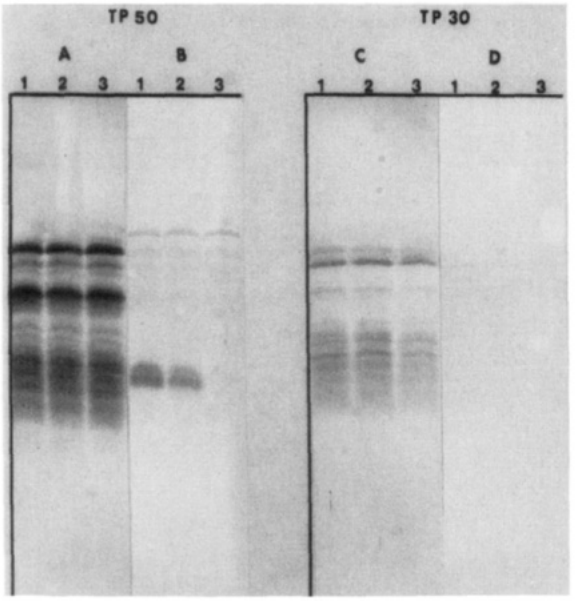

FIGURE 7: Affinity labeling of ribosomal subunits by a virginiamycin S derivative. Ribosomes bearing covalently linked HSE derivative of virginiamycin S (samples I–III of Table II) were dissociated. Ribosomal proteins obtained by dissociation of 30S and 50S subunits were fractionated and analyzed as in the legend to Figure 5. Left panel (TP50) and right panel (TP30) are ribosomal proteins from 50S and 30S subunits, respectively. Protein patterns: parts A (60 μg of TP50) and C (35 μg of TP30), after India ink staining; parts B and D, after immunodetection.

Wittman (1970) procedure of bidimensional electrophoresis. A better resolution of the crowded region was thus obtained. Label was found to be confined to a double spot (indicated by arrows in Figure 8E), which overlapped proteins L18 and L22 of the reference pattern (Figure 8D,F).

Discussion

Choice of an Affinity-Labeling Reagent. Few antibiotic derivatives were successfully used for affinity labeling of ribosomes. Main limitations of this approach are a lack of biological activity of modified antibiotics and the aspecific reactivity of the grafted reactive groups. In our case, the virginiamycin S moieties respectively susceptible or unsusceptible to noninactivating modifications were previously identified. Involvement of the picolinyl ring (Figure 1) in fixation to ribosomes was supported by the spectrofluorometric studies of the virginiamycin S binding reaction to 50S (Parfait et al., 1978; Moureau et al., 1983; Di Giambattista et al., 1984, 1987) and by the fact that all known reported modifications of this part of the molecule abolished its antibiotic action. By contrast, the 4-oxopipecolinic acid component, which is replaced by some amino acids (aspartic acid in vernamycin C and doricin and proline in patricin A) in other type B synergimycins [cf. Cocito (1979) for review], is a dispensable part of the molecule. The keto group of the 4-oxopipecolinic acid component of virginiamycin S was thus chosen for the grafting of a reactive arm.

Preparation and Properties of an Antibiotic Derivative for Affinity Labeling of Ribosomes. Contrarily to most derivatives at the oxopipecolic ring, which are unstable, oxime derivatives in Table I, such as the carboxyl derivative (virginiamycin S–COOH) and the succinimide ester (HSE), are stable (Patent 08701099). These compounds had biological and biochemical activities comparable to that of virginiamycin S (cf. binding curves in Figure 3 and association constants in Table I), and stoichiometry of the binding reaction was 1 mol of antibiotic/mol of ribosome. The HSE derivative of virginiamycin S carried a succinimidyl group able to react with the ϵ-NH_2 group of protein lysine and to minor extent with the NH group of histidine and SH group of cysteine. The affinity-labeling experiments in Figures 5 and 7 proved the HSE derivative of virginiamycin S to be a selective L protein reagent. Indeed, its binding to the 30S subunit is excluded by the experiments in Figure 7.

Binding Conditions of the HSE Derivative to Ribosomes. Specificity of binding of the HSE derivative of virginiamycin S to ribosome can be controlled by the use of erythromycin, which prevents the fixation of the former antibiotic (Parfait et al., 1978; Moureau et al., 1983). Indeed, erythromycin inhibited by more than 80% the reaction between HSE and ribosome (Table II). In affinity-labeling experiments, this macrolide was used to remove noncovalently bound HSE from HSE–ribosome complexes prepared at 30 °C. Consequently, the binding reaction of HSE to ribosome was reversible at 4 °C and irreversible at 30 °C.

Because of the synergistic effect of A and B synergimycins (the association constant of the reaction of virginiamycin S–ribosome complex formation undergoes a 10-fold increase in the presence of its partner), one would expect a larger amount of linked HSE in sample containing virginiamycin M. However, fluorometric measurements in Table II and immunoenzymometric evaluation in Figures 5 and 6 yielded unmatched values. Such a discrepancy can be accounted for by the difference in the experimental conditions: noncovalently bound HSE was removed from ribosome by erythromycin in the experiments described in Figure 5, but not in those of Table II. Note that erythromycin displaces virginiamycin S from ribosomes only in the absence of virginiamycin M (Parfait et al., 1981; Parfait & Cocito, 1980) and that the action of the latter is inapparent in the presence of a saturating concentration of virginiamycin S.

Immunological Reactivity and Specificity of Anti-Virginiamycin S Immunoglobulins. Induction of antibody synthesis against haptens coupled to immunogen vectors is a widely used immunological procedure. In the present work, the HSE–albumin conjugate was injected to rabbits for antiserum production. In addition, the carboxyl derivative for virginiamycin S grafted to aminoalkylagarose was used as a support for affinity gel chromatography. Immunoglobulins purified by the two-step procedure outlined under Materials and Methods proved to react with virginiamycin S and not with its carrier (Figure 4). Consequently, they must be considered as highly specific reagents.

Identification of the Proteins at the Virginiamycin S Binding Site. Proteins from covalent HSE–ribosome complexes have been first fractionated by the two-dimensional electrophoresis procedure of Mets and Bogorad (1974), a fast microanalytical method allowing efficient transblotting to nitrocellulose membranes. Unfortunately, this procedure yielded poor resolution of HSE-labeled proteins. The increase in molecular weight (about 1000 according to Table I) and the decrease of migration velocity (one ϵ-NH_3^+ esterified) resulting from covalent linkage of HSE to a protein is expected to reduce its R_f in both dimensions. Hence, proteins L18, L19, L22, and L24 ought to be considered possible candidates (Figure 8A–C). The Kaltschmidt and Wittman (1970) procedure afforded better resolution of the crowded region, but yielded low transblotting efficiency. The elongated spot in Figure 8E appears to correspond to proteins L18 and L22 either unmodified or modified by covalently bound HSE (i.e., four components, two of which displaced upward and leftward) (reference to Figure 8D,F). Although the mapping of L22

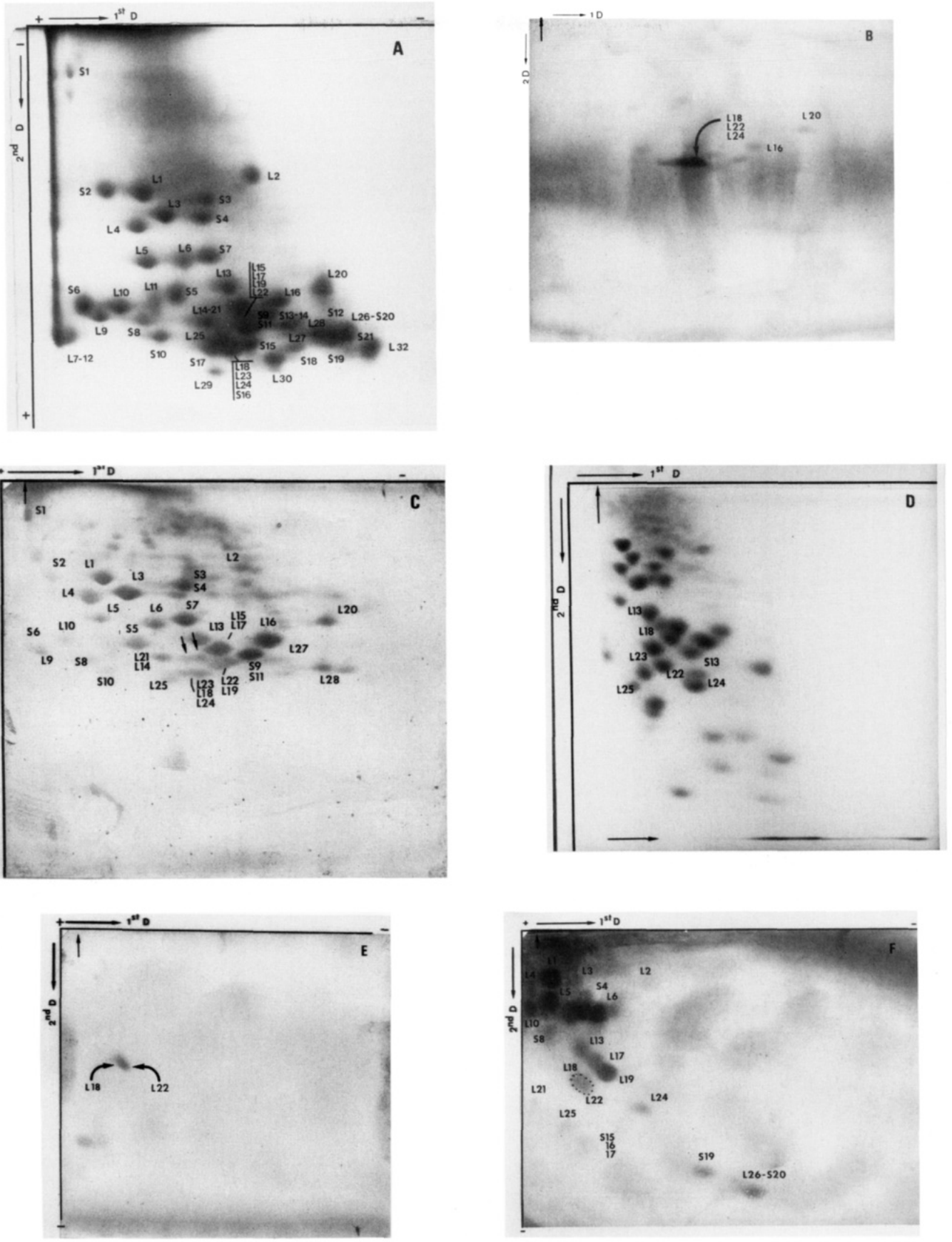

FIGURE 8: Affinity labeling of ribosomal proteins by a virginiamycin S derivative. Covalent ribosome–virginiamycin S complexes, affinity labeled by the HSE derivative (as indicated under Materials and Methods and in Table II, sample I), were dissociated, and proteins were fractionated by polyacrylamide gel two-dimensional electrophoresis, according to the procedures of Mets and Bogorad (1974) (samples A–C) and of Kaltschmidt and Wittmann (1970) (samples D–F). Proteins were transblotted to two nitrocellulose sheets, which were submitted respectively to India ink staining (samples C, F) and to immunorelevation with anti-viriginiamycin S IgG (samples B, E). Patterns A and D were the Coomassie blue stained polyacrylamide gels. Arrows point to immunodetected proteins, as does the dotted spot in panel E.

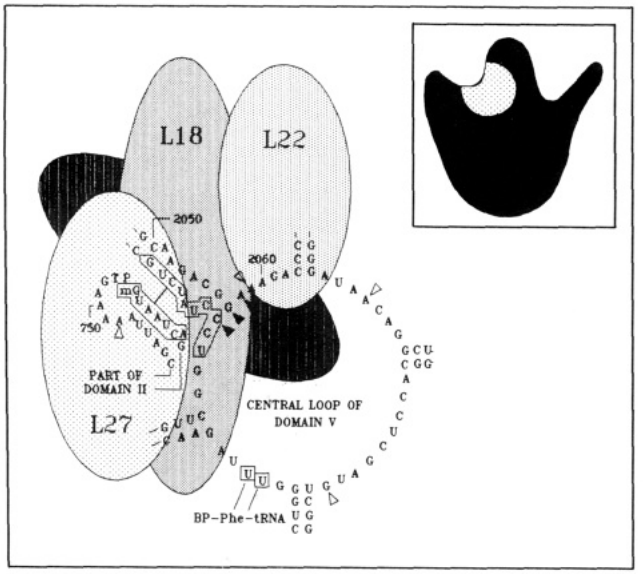

FIGURE 9: Topological model of the binding site for MLS antibiotics on the 50S ribosomal subunit. Ribosomal proteins (L15, L18, L22, and L27), which were affinity labeled by different MLS antibiotics and located by immune electron microscopy, and bases in domains II and V of 23S rRNA, which were altered by MLS^R mutations (▲) or protected (△) by MLS antibiotics, are depicted. Segments of domain II, which were joined by inter-cross-linking, and bases of domain V, which were cross-linked to a derivative of Phe-tRNA, are encircled. The dotted area in the insert indicates the consensus topological situation of the peptidyltransferase domain, which is overlapped by the MLS binding site.

on ribosome surface has not been realized, cross-linking of L22 and L18 has been recently obtained (Redl et al., 1989). The low affinity labeling of proteins L16, L20, and S5 (Figure 8B) was presumably aspecific, being insensitive to erythromycin during the labeling step (not shown).

Binding of MLS Antibiotics to Ribosomes. Affinity labeling by aminoacyl-tRNA at the ribosomal A site has pointed to proteins L2, L15, L16, and L27 and that with P site-bound aminoacyl-tRNA to L2 and L27 (Pellegrini & Cantor, 1977). Moreover, analysis of macrolide-resistant mutants and affinity labeling of ribosomal proteins by these antibiotics have shown the involvement of L27 (Tejedor & Ballesta, 1986), L18 (Siegrist et al., 1985), L22 and L4 (Wittmann et al., 1973; Pardo & Rosset, 1977; Arévalo et al., 1988), and L15 (Hummel et al., 1979; Arévalo et al., 1988). Two of the macrolide-binding L proteins correspond to those identified in the present work as virginiamycin S labeled proteins. In agreement with such a conclusion are previous studies indicating the overlapping of virginiamycin S binding sites and erythromycin-binding sites within the peptidyltransferase domain (Di Giambattista et al., 1987). The former binding site has been recently located, by nonradiant energy transfer, at the base of the central protuberance (Di Giambattista et al., 1986), the presumptive location of the peptidyltransferase center. To this region, most of the above-mentioned proteins (L2, L15, L16, L18, L22, L27) have been assigned by immune electron microscopy and cross-link experiments (Stöffler & Stöffler-Meilicke, 1986; Redl et al., 1989). Although the removal of protein L16 was found to suppress virginiamycin S binding, and its addition to restore this ability of stripped cores (de Béthune & Nierhaus, 1978), there is no proof for a fixation of this antibiotic to L16, which in fact plays a key role in ribosome conformation.

Relationship between Antibiotic Binding Sites, Mapping of Antibiotic Resistance Mutations, and the Peptidyltransferase Domain. Evidence has now been provided for the presence of domains II and V of 23S rRNA within the peptidyltransferase center. Dimethylation of adenine A 2058 in 23S rRNA was found to be responsible for the MLS^R ("undissociated resistance") phenotype (Lai & Weisblum, 1971) and for ribosome "shielding" of erythromycin-producing streptomycetes (Fujisawa & Weisblum, 1981; Skinner et al., 1983; Uchyama & Weisblum, 1985). Mutagenization of rrnH genes leading to single changes in positions G 2057, A 2058, and C 2611 resulted in "dissociated" resistance to single MLS antibiotics (Sigmund & Morgan, 1982; Sigmund et al., 1984; Sor & Fukuhara, 1982, 1984; Ettayebi et al., 1985; Vester & Garrett, 1987). Moreover, macrolides protected bases 2058–2062, chloramphenicol bases 2059–2062, and vernamycin B (a type B synergimycin) bases 2058–2062 of domain V and A 752 of domain II in 23S rRNA (Moazed & Noller, 1987). On the other hand, proteins L15 and L16 were required for erythromycin binding (Teraoka & Nierhaus, 1978).

As pointed out by Cundliffe (1987), there is no well documented case of antibiotic resistance mutation, entailing the alteration of a protein, located within the corresponding antibiotic binding site. It is equally difficult, however, to postulate that a single base change in 23S rRNA produces the MLS^R phenotype because A 2058 is the receptor for a number of unrelated antibiotic molecules. A more reasonable hypothesis is that the peptidyltransferase catalytic center, which is formed by two domains of 23S rRNA tightly interacting with a series of L proteins, is overlapped by the binding sites of different antibiotics. A transition in 23S rRNA might induce a conformational alteration of ribosomes resulting in a lack of MLS binding.

We propose that proteins L18 and L22, affinity labeled at the virginiamycin S binding site, in conjunction with proteins L15 and L27 and domains V and II of 23S rRNA, are parts of the catalytic center of peptidyltransferase. This model is illustrated by the scheme displayed in Figure 9, which takes into account literature data on in vivo and in vitro produced mutations, protection experiments, and affinity-labeling works. Our model is supported by numerous observations including the interaction of L18 with domain V (Brimacombe et al., 1983) and that of L22 with domain II of 23S rRNA (Chen-Schmeisser & Garrett, 1976), the affinity labeling of L18 with peptidyl-tRNA derivatives (Hsiung et al., 1974), the intramolecular cross-link between domains II and V (Stiege et al., 1983), and the affinity labeling of U2584 and U2585 with a benzophenone derivative of Phe-tRNA (Barta et al., 1984).

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