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Photoaffinity Labeling of the Pactamycin Binding Site on Eubacterial Ribosomes†

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ABSTRACT: Pactamycin, an inhibitor of the initial steps of protein synthesis, has an acetophenone group in its chemical structure that makes the drug a potentially photoreactive molecule. In addition, the presence of a phenolic residue makes it easily susceptible to radioactive labeling. Through iodination, one radioactive derivative of pactamycin has been obtained with biological activities similar to the unmodified drug when tested on in vivo and cell-free systems. With the use of [^{125}I]iodopactamycin, ribosomes of *Escherichia coli* have been photolabeled under conditions that preserve the activity of the particles and guarantee the specificity of the binding sites. Under these conditions, RNA is preferentially labeled when free, small ribosomal subunits are photolabeled, but proteins are the main target in the whole ribosome. This indicates that an important conformational change takes place in the binding site on association of the two subunits. The major labeled proteins are S2, *S4*, S18, *S21*, and L13. These proteins in the pactamycin binding site are probably related to the initiation step of protein synthesis.

The study of the mode of action of antibiotics has the 2-fold interest of opening up possibilities to potentiate their therapeutic activity and of providing important data that can help us to understand the structure and activity of their biological targets.

Pactamycin, a drug initially developed as a potential antitumor agent, is especially interesting as an inhibitor of protein synthesis in eukaryotic and prokaryotic systems (Goldberg, 1974). It blocks the initial steps of the protein synthesis process [for a review, see Vázquez (1979)] either by interfering with the association of the large ribosomal subunit with the 40S initiation complex or by blocking the ribosome after the formation of the first peptide bond (Kappen & Goldberg, 1976). In any case, the drug binds preferentially to the small subunit and has lower affinity for the whole riosome and practically none for the large subunit when tested at low inhibitory concentrations (McDonald & Goldberg, 1970).

The identification of the ribosomal components involved in the antibiotic binding site is an important step in understanding its mode of action. Affinity labeling techniques are probably the most direct method to approach this problem and, in fact, have been used fruitfully in the case of many ribosomal effectors (Cooperman, 1980). The applicability of this technique is usually restricted to cases where radioactively labeled and chemically reactive derivatives are available. Pactamycin, having in its molecule a potentially photoactive (Cowan & Drisko, 1978) acetophenone group, as well as a phenolic group susceptible to radioactive iodination, has the appropriate conditions for the identification of the components of its ribosomal binding site using photoaffinity procedures.

The identification of these components in *Escherchia coli* ribosomes would allow us, considering the activity of the drug, to establish correlations between ribosomal components involved, directly or indirectly, in the initiation step of protein synthesis.

Materials and Methods

Iodination of Pactamycin. Pactamycin (25–50 μg) in 50 mM phosphate, pH 7.5, and 50% ethanol was treated with 0.5–0.6 mCi of Na^{125}I by a modification of the chloramine T method that avoids direct contact between the oxidant compound and the drug, as reported previously (Tejedor & Ballesta, 1982).

After treatment, the reaction mixture was separated by an LH20 Sephadex column (0.9 × 20 cm), equilibrated, and eluted with 96% ethanol. Fractions (0.5 mL) were collected and checked in a γ counter. The fractions containing the drug were pooled, evaporated, dissolved in 200 μL of Cl_2CH_2, and

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finally separated by thin-layer chromatography (TLC)[1] on silica gel using Cl_2CH_2–CH_3OH (17:2) as eluant. The position of the radioactive spots was localized by autoradiography, eluted from the plate by ethanol or methanol, and stored at −20 °C. The purity of the eluted spots was tested by TLC.

Nonradioactive iodopactamycin was prepared, using the chloramine T method, in 600 μL of 166 mM phosphate buffer, pH 7.5, and 66% ethanol containing 3.7 mg of drug and 10 mg of KI mixed with 200 μL of chloramine T (10 mg/mL). The treatment lasted for 25 min at room temperature, and the reaction was stopped by adding 100 μL of Na_2SO_3 (40 mg/mL). After elimination of the ethanol by evaporation, the mixture was extracted 4 times with 0.5 mL of ethyl acetate. The extract, dried over anhydrous Na_2SO_4, was resolved by TLC as reported for the radioactive derivative.

Inhibition of Bacterial Growth. Escherichia coli MRE-600 was grown in yeast extract–peptone–glucose medium on agar plates. Antibiotics were placed on filter paper disks on the surface of the plates, and inhibition was estimated by the diameter of the inhibition halo.

Binding of Pactamycin to Ribosomes. Binding of [^{125}I]-iodopactamycin was checked either by filtration through Millipore filters (Stewart & Goldberg, 1973) or by centrifugation through sucrose gradients. In the latter case, 100 μL of 0.5–0.75 μM ribosomes in 10 mM Tris-HCl buffer, pH 7.4, 60 mM NH_4Cl, 10 mM $MgCl_2$, and 6 mM 2-mercaptoethanol, kept at 0 °C in the presence of 0.75–1.125 μM pactamycin, was centrifuged through 5-mL sucrose gradients as specified in the text.

Ribosomes and ribosomal subunits were prepared according to standard methods (Staehelin & Maglott, 1971).

Photolabeling of Ribosomes with Iodopactamycin. Ribosomes [(1–1.2) × 10^{-6} M], extensively dialyzed against 10 mM sodium borate, pH 7.0, 100 mM KCl, and 10 mM $MgCl_2$, were incubated in the presence of 1.5 × 10^{-7} M [^{125}I]iodopactamycin at 0 °C for 20 min and then irradiated in a refrigerated bath, to keep the sample temperature below 10 °C, at 5 cm with a medium-pressure 125-W mercury lamp covered with a borosilicate filter.

Covalent incorporation of radioactivity was followed by precipitation with 10% trichloroacetic acid at 0 °C and filtration on glass fiber filters. The filtration must be performed rapidly after precipitation since slow loss of radioactivity has been detected when the precipitated samples are left, even at 0 °C.

When required, irradiated ribosomes were precipitated with 10% poly(ethylene glycol) 6000 and collected by centrifugation. The pelleted ribosomes were resuspended in one-sixth of the original volume of 10 mM Tris-HCl, pH 7.4, 60 mM NH_4Cl, 1 mM $MgCl_2$, and 5 mM β-mercaptoethanol and separated by centrifugation through 5–20% sucrose gradients in the same buffer using a SW27 Beckman rotor for 14.6 h at 20 K. The gradients were fractionated, and the A_{260} and the total and trichloroacetic acid precipitable radioactivities were checked in every fraction.

Electrophoretic Methods. Ribosomal proteins and RNA were separated by the urea–LiCl method (Traub et al., 1971). When recovery of RNA was not attempted, ribosomes were treated with a mixture of RNase A and RNase T1 as described (León-Rivera et al., 1980).

The proteins were separated either by one-dimensional SDS gel electrophoresis (Weber & Osborn, 1969) or by two-dimensional polyacrylamide gel electrophoresis (Kaltschmidt & Wittmann, 1970).

After electrophoresis, gels were stained with 0.4% Coomassie blue in 50% ethanol for 1–2 h in the absence of acetic acid and then kept in 60% acetone for 2 h at 0 °C. Destaining was performed in 25% ethanol at room temperature overnight.

Dried slabs were exposed on Kodak X-O-matt radiographic plates to detect the labeled spots. Quantification of spots was carried out by using a Photomation P-1700 densitometer from Optronics connected to a Pdp-11 computer from Digital.

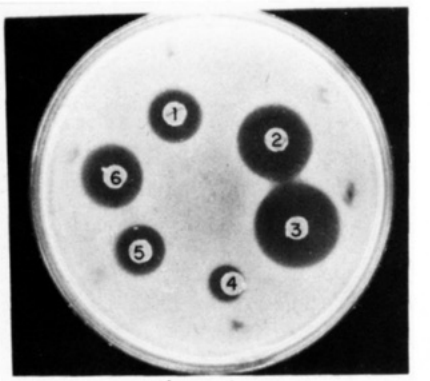

FIGURE 1: Inhibition of *E. coli* growth: 2, 5, and 10 μL of 10^{-4} M pactamycin (wells 1, 2, and 3) and iodopactamycin (wells 4, 5, and 6) were placed in the respective disks to inhibit cell growth in the agar plate.

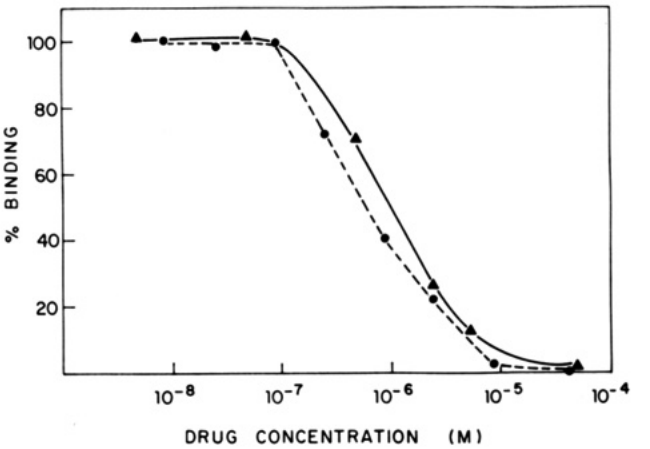

FIGURE 2: Competition of pactamycin and [^{125}I]iodopactamycin for binding to ribosomes. 0.24 μM ribosomes of *E. coli* were incubated with 0.35 μM [^{125}I]iodopactamycin in the presence of increasing concentrations of either unmodified pactamycin (▲) or nonradioactive iodopactamycin (●) under the ionic conditions described under Materials and Methods and filtered through nitrocellulose filters. 100% binding corresponds to 0.6 mol/mol of ribosomes.

RESULTS

Preparation of Iodopactamycin. The iodination of pactamycin under the mild conditions used results in the presence of four radioactive compounds (A, B, C, and D) when the treated samples are resolved by TLC. The low yield of spots C and D, and the low biological activity of B, led us to concentrate our work on compound A only.

The specific radioactivity of [^{125}I]iodopactamycin A, measured spectroscopically and by isotopic dilution using a nonradioactive derivative, was 2.7 Ci/mmol.

Biological Activities of Iodopactamycin. Pactamycin efficientlty inhibits cell growth by blocking cellular protein synthesis. Iodopactamycin A has an inhibitory activity similar to that of unmodified pactamycin in *E. coli* growth (Figure 1) as well as in a S-30 cell-free system from reticulocytes (data not shown).

[1] Abbreviations: SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

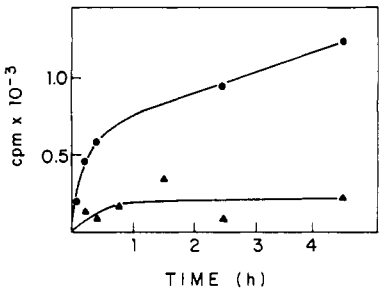

FIGURE 3: Kinetics of iodopactamycin covalent binding to 70S ribosomes. 1.0 μM 70S ribosomes of *E. coli* dialyzed against 10 mM borate, pH 7.0 (●) and 8.0 (▲), 10 mM $MgCl_2$, and 100 mM KCl were treated with 0.22 μM [^{125}I]iodopactamycin and irradiated at 4 °C as described under Materials and Methods. 50-μL aliquots were taken at the indicated times, precipitated with 5% trichloroacetic acid, and filtered through glass fiber filters. The background radioactivity at zero time was 1280 cpm and was subtracted from all the samples.

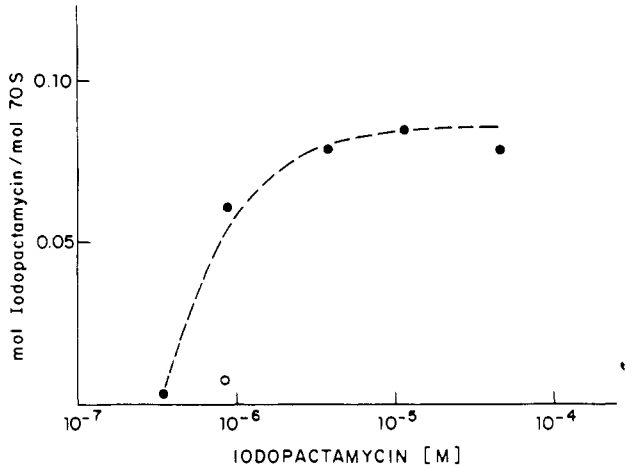

FIGURE 4: Covalent binding of [^{125}I]iodopactamycin in 70S ribosomes as a function of drug concentration. 1 μM ribosomes from *E. coli* were irradiated as described under Materials and Methods with increasing concentrations of isotopically diluted [^{125}I]iodopactamycin (specific activity 223 cpm/pmol) for 3 h at 4 °C. 50-μL aliquots were precipitated with 10% trichloroacetic acid and 0 °C and filtered through glass fiber filters, dried, and counted (●). Samples under the same conditions but without irradiation were used as blanks, and their radioactivity was subtracted from the respective irradiated samples. A duplicate of the 0.85 μM iodopactamycin sample containing, in addition, 10 μM pactamycin was also included (○).

Iodopactamycin A competes with the unmodified drug for the same ribosomal binding site (Figure 2). Moreover, the competition curves of [^{125}I]iodopactamycin with unmodified pactamycin and nonradioactive iodopactamycin are very similar (Figure 2), indicating that the affinity of both compounds for the binding site is similar.

Covalent Binding of [^{125}I]Iodopactamycin to Ribosomes. Irradiation of iodopactamycin A in the presence of ribosomes, under conditions that inactivate no more than 15% of the ribosomes, induces the incorporation of radioactivity covalently bound to the particles as a function of the time of irradiation (Figure 3). This incorporation is strongly dependent on the pH of the irradiated sample (Figure 3) and is slowly hydrolyzed if left standing in trichloroacetic acid even at 0 °C.

The covalent incorporation of pactamycin into ribosomes is a function of the drug concentration in the reaction mixture and seems to take place at specific sites since a saturation plateau is reached at low pactamycin to ribosome ratios (Figure 4).

The specificity of the covalent binding is confirmed by the competition effect produced by pactamycin in the reaction. A 10-fold excess of drug practically abolishes the covalent incorporation of radioactivity into the ribosomes (Figure 4).

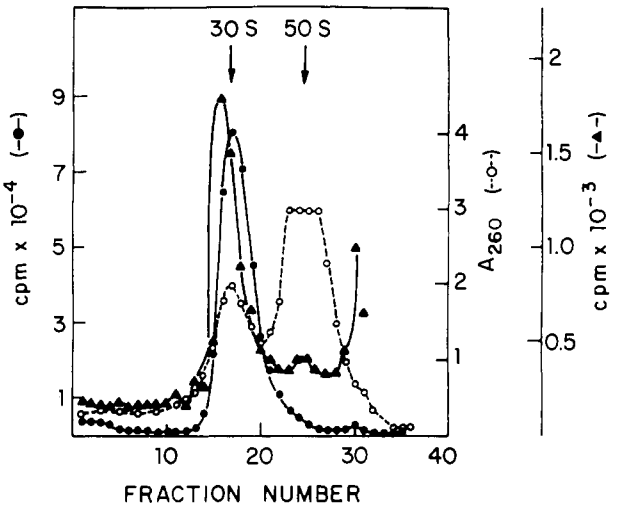

FIGURE 5: Total and covalent binding of [^{125}I]iodopactamycin to *E. coli* ribosomes. 1 μM ribosomes dialyzed against 10 mM borate, pH 7.0, were irradiated in the presence of 1.6 μM [^{125}I]iodopactamycin. After irradiation, the particles were precipitated with poly(ethylene glycol), redissolved in a small volume of buffer, and spun down through 5–20% sucrose gradients as indicated under Materials and Methods. The gradient was fractionated, and the fractions were checked for A_{260} (○), total radioactivity (●), and trichloroacetic acid precipitable radioactivity (▲).

Table I: Covalently Bound Radioactivity in Ribosomal Components after Irradiation with [^{125}I]Iodopactamycin[a]

	radioact. (cpm) in *E. coli*	
	small subunit	whole ribosome
rRNA	2830	2447
protein	1231	3610
RNA/protein ratio	2.3	0.7

[a] Either small subunits or whole ribosomes were irradiated under the conditions described in Figure 6 and under Materials and Methods. Ribosomal proteins and RNA were separated by the LiCl–urea method, and the radioactivity associated with each fraction was measured.

Distribution of Radioactivity in the Ribosome. Photolabeled ribosomes, separated by sucrose gradient centrifugation, indicate that 2–5.5% of the total radioactivity is covalently associated with the ribosome. Most of this radioactivity is bound to the 30S subunit, but a small, although reproducible, fraction appears in the large subunit (Figure 5).

Distribution of the covalently bound radioactivity between ribosomal proteins and RNA was checked. As shown in Table I, there is a remarkable difference in this distribution when tested in whole ribosomes and in purified small subunits, suggesting the existence of either a conformational change or a shielding effect in the binding site upon subunit association.

Identification of Ribosomal Proteins Labeled by Iodopactamycin. Ribosomal proteins extracted from particles irradiated for increasing periods of time were separated by SDS–polyacrylamide gel electrophoresis in order to test whether the time of irradiation changes the pattern of labeling of proteins. Figure 6 shows that this is not the case, and a similar pattern is obtained after irradiation from 1 to 3 h. These results indicate the absence of secondary reactions due to photoproducts of the drug that could have labeled different sites, as has been shown in other instances (Goldman et al., 1983).

Separation by two-dimensional gel electrophoresis of the labeled proteins followed by autoradiography of the plates allowed us to identify the individual labeled spots. In Figure 7, results of two-dimensional gel electrophoresis of labeled

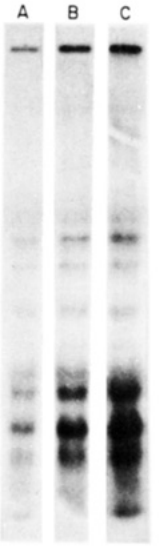

FIGURE 6: Proteins of 70S *E. coli* ribosomes photolabeled with [^{125}I]iodopactamycin. 1.2 μM 70S ribosomes were irradiated with 2 μM [^{125}I]iodopactamycin as in Figure 8 for 1 (A), 2 (B), and 3 h (C). After irradiation, 1 volume of buffer with 2% SDS was added. The samples were incubated at 80 °C for 10 min and then directly applied to the gel for SDS electrophoresis. The spots were detected by autoradiography.

proteins from 30 subunits are presented. Similar results are obtained in the case of 70S ribosomes. Spots on the autoradiogram coincide with spots in the stained gel so that the identification of the labeled proteins is not difficult.

Quantification of the data was performed by densitometry, and the results are presented in Table II.

Table II: Quantification of Labeling in *E. coli* Ribosomal Proteins[a]

protein	% small subunit	% whole ribosome
S18	32.6	16.2
S21	20.5	
S4	15.2	26.0
S2	11.3	
S13	7.7	8.8
S9	6.9	7.2
S15	5.6	9.1
L13		15.6
L4		7.6
L6/S5		6.0
L2		3.5

[a] Proteins were extracted from either 30S subunits or 70S ribosomes independently irradiated in the presence of [^{125}I]iodopactamycin as indicated in Figure 7 and separated by two-dimensional gel electrophoresis. Autoradiograms from the gels were quantified by a computer program for analysis of the densitometer scan. The data are normalized as percentages of the total optical density of the spots.

Discussion

The results presented in this report indicate that pactamycin can be radioactively labeled by iodination of the phenolic group present in its structure, obtaining several derivatives, one of which, [^{125}I]iodopactamycin A, is as active as the unmodified drug.

The presence, in the pactamycin molecule, of an aromatic ketone makes this drug potentially photoreactive, and therefore a good candidate for affinity labeling studies. The results of irradiation of the drug in the presence of the ribosome show that covalent binding of [^{125}I]iodopactamycin takes place and does so at specific binding sites, as deduced from the saturation and competition experiments. Therefore, the conditions are suitable for identification of the ribosomal components of this binding site, after affinity labeling experiments.

The results obtained indicate, first of all, that the ratio of label incorporated into protein and RNA changes drastically upon association of the subunits. Also, the pattern of protein labeling changes in the two particles, as is clear in the case of proteins S2 and S21, two of the most labeled proteins in the 30S subunit, labeled at a very low level in the whole ribosome. On the other hand, protein S4 labeling increases in the 70S particles. These data indicate that subunit association seriously affects the structure of the binding site although a shielding effect might also take place. The binding site of pactamycin seems to be localized on the interface between both subunits, since, although the large subunit does not bind the drug directly, some of its components become labeled in the whole ribosome. This situation has also been found in affinity labeling studies of other antibiotics, namely, pleuromutilin (Hogenauer et al., 1981), puromycin (Nicholson et al., 1982a,b), streptomycin (Leŏn-Rivera, 1981), and macrolides (Tejedor & Ballesta, 1985).

In the case of the small subunit, protein S18 is the most heavily labeled protein, followed by S4 and S2. Proteins S13, S15, and S21 have considerably less labeling, and S3 and S9 have only traces of radioactivity. Protein S18 is a very reactive polypeptide, probably due to the presence of a highly exposed cysteine group (Kang et al., 1974), and its labeling by affinity reagents might be a subject of controversy. However, in many cases, this protein has been localized with confidence as a component of ribosomal active centers (Ofengand, 1980). In our case, the binding conditions used, together with the fact that the labeling photoreaction does not seem to involve nu-

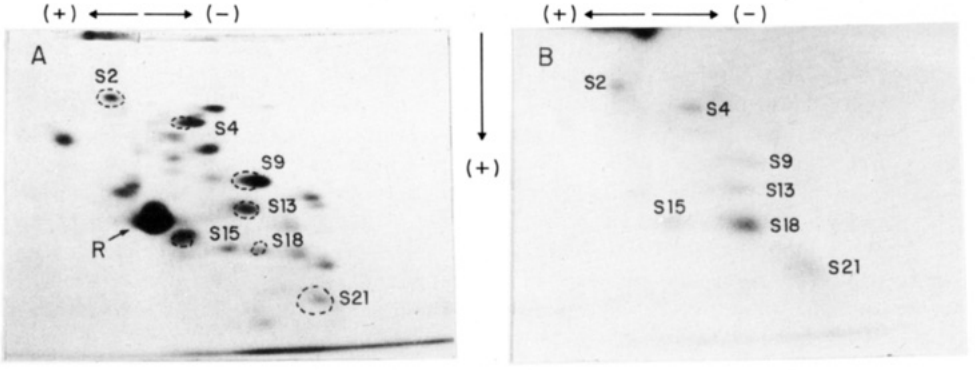

FIGURE 7: Two-dimensional gel electrophoresis of [^{125}I]pactamycin-labeled 30S ribosomal proteins (left) and their corresponding autoradiograms (right). Irradiation was performed as in Figure 6. After irradiation, ribosomes were treated with RNase T1 and RNase A, and the proteins were precipitated with 5 volumes of acetone at −20 °C, dissolved in the appropriate buffer, and separated by two-dimensional electrophoresis. The radioactive spots were detected by autoradiography. The number of the labeled proteins are marked in the autoradiogram and in the stained gels where the positions of the labeled spots have also been circled. R indicates the position of RNase used for protein preparation (see Materials and Methods).

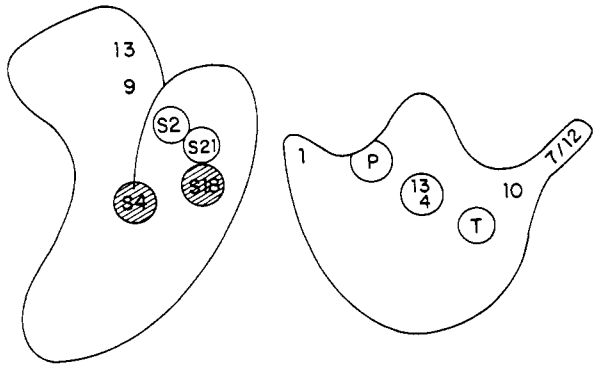

FIGURE 8: Model of *E. coli* 30S (left) and 50S (right) ribosomal subunits. The positions of the proteins have been taken from immunoelectronmicroscopy data [for a recent review, see Wittmann (1983)] as well as from cross-linking and affinity labeling results (Boileau et al., 1983). In the 30S model, batched circles correspond to the most labeled proteins in 30S and 70S particles and open circles to major labeled proteins in 30S particles only, and uncircled numbers are proteins labeled in minor proportions. The proteins included in the 50S model correspond to well-characterized markers. The circled number is the suggested localization of the proteins labeled by pactamycin in the 50S subunit, deduced from the position of the corresponding proteins labeled in the 30S particle and following the model of subunit association proposed by Lake (1982). P and T correspond to the puromycin and thiostrepton binding sites, respectively [see Wittmann (1983)].

cleophilic reagents, make us fairly confident of the specificity of S18 labeling. Another point in favor of this specificity is the fact that S18 is considerably less labeled in the 70S ribosome than in the small subunit, since the labeling of S18 by unspecific reagents does not seem to be affected by subunit association (Michalski & Sells, 1975).

All the labeled proteins have been localized on the interface region of the ribosome by protein–protein and protein–RNA cross-linking (Coyer et al., 1981; Chiam & Wagner, 1982). On the other hand, cross-linking data also indicate that most of them form a cluster, since they are cross-linked either between themselves (S4–S9, S18, S13–S4, L4–S13) or to proteins in the same group, namely, S3, S5, S8, L1, and L14 (Bollen et al., 1975; Traut et al., 1980; Cover et al., 1981; Lambert & Traut, 1981). Five of them (S2, S18, S13, and S21) are cross-linked to one or more initiation factors (Cooperman et al., 1981; Pon et al., 1982; Bioleau et al., 1983), and by affinity labeling techniques, proteins S4, S18, S13, and S21 have been involved in the mRNA binding site on the ribosome (Ofengand, 1980).

Some of the labeled proteins have been related also to the interaction site of other antibiotics that affect the initiation of protein synthesis. Thus, protein S13 seems to be involved in streptomycin interaction (Leôn-Rivera, 1981), proteins S4 and L13 in the tobramycin site (Tangy et al., 1983), proteins S18 and S21 in pleuromutilin inhibition (Hogenauer et al., 1981), and protein S2 in kasugamycin resistance (Okuyama et al., 1974).

When the position of the major labeled proteins is located in the model of the 30S subunit according to data from different authors (Figure 8), the binding site of pactamycin can be placed in the base of the cleft between the platform and the body of the subunit, close to the region where the initiation factors seem to interact (Boileau et al., 1983; Schwartz et al., 1983) and where the mRNA also binds to the particle (Zimmerman et al., 1979).

At present, we do not have data available to find the position of the proteins labeled in the large subunit, namely, L4 and L13. Our results, however, suggest that these two proteins must be located in the 50S subunit interface opposite the mRNA binding site. In the model of subunit association proposed by Lake (1982), this site is on the seat of the subunit, somewhere between the puromycin and chloramphenicol binding sites and the thiostrepton site (Wittman, 1983) (Figure 8). This position fits well with some cross-linking data (Traut et al., 1980) indicating that protein L4 is close to L11 and L13 is close to L10.

These data agree with the postulated mode of action of pactamycin at the initiation steps of protein synthesis, probably interfering with the correct binding of the large subunit to the initiation 40S complex.

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Purification and Characterization of a Non-Vitellogenin, Estrogen-Induced Plasma Protein from the American Bullfrog *Rana catesbeiana*†

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ABSTRACT: A non-vitellogenin, estrogen-induced protein has been detected for the first time in the plasma of male *Rana catesbeiana*. A >90% purification of this plasma protein was achieved by salt fractionation with Mg(II) followed by ion-exchange chromatography on DEAE- and CM-cellulose. Immunoelectrophoretic analysis with various antisera showed no immunological cross-reactivity between this protein and vitellogenin. The molecular mass of the purified protein was determined to be 116 000 daltons by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and 105 000 daltons by analytical ultracentrifugation. Sedimentation studies indicate the protein is a nonaggregating spherical monomer with a sedimentation coefficient of 7.5 S. Amino acid analysis demonstrated a composition different from that of vitellogenin and lipovitellin A. Limited proteolysis with trypsin, chymotrypsin, and *Bacillus subtilis* protease revealed no common peptides on SDS-polyacrylamide gels. Phosphate analysis indicated that, on a molar basis, the non-vitellogen, estrogen-induced protein had ≤3% of the phosphate found in vitellogenin. Further studies of the structure, function, and metabolism of this protein may reveal information relating to the hormonal control of vitellogenesis.

Vitellogenin (Vg) is a plasma protein synthesized by the liver of female oviparous vertebrates in response to seasonal increases of estrogen. During transit through the hepatocyte, the Vg is glycosylated and acquires noncovalently bound lipid. The Vg secreted by the liver is removed from the bloodstream and subsequently processed by oocytes into the egg yolk proteins phosvitin and lipovitellin (Bergink & Wallace, 1974). The hormonal regulation of vitellogenesis has been widely studied in avian and amphibian species [e.g., see Tata & Smith (1979) and Banaszak et al. (1982)]. Amphibians offer specific advantages in that they undergo a metamorphosis during development and do not require calcium mobilization for egg shell production during the vitellogenic response, and being poikilotherms, the metabolic events associated with vitellogenesis occur over a longer period of time.

Wallace & Jared (1968) demonstrated that vitellogenesis can be induced in male frogs, which do not normally synthesize Vg, by a single estrogen injection. Such an approach allows one to measure the time sequence and magnitude of the molecular events associated with vitellogenesis against a zero background. For example, Baker & Shapiro (1977) have shown that the mRNA for Vg is undetectable in the liver of unstimulated male *Xenopus laevis*, but following estrogen administration, the mRNA Vg increases at a linear rate for 12 days to >30 000 copies per cell. As a consequence of the high levels of mRNA, large amounts of Vg are synthesized and secreted into the plasma. In male animals, Vg accumulates because it cannot be sequestered and processed by oocytes. Vg levels of 40-60 mg/mL of plasma have been demonstrated in male frogs 10-14 days after a single estrogen injection (Follett & Redshaw, 1968; Wallace, 1970; Hess, 1981).

Most studies of amphibian vitellogenesis have utilized the South African clawed toad *Xenopus laevis*. In our laboratory, we have used the American bullfrog *Rana catesbeiana* to investigate the metabolism of plasma proteins. We have purified bullfrog Vg by two independent methods and have shown that it contains two peptides having molecular masses of 185 000 and 195 000 daltons (Da) (Hess, 1981). Vitellogenin purified from *Xenopus* has a similar molecular mass (Wiley

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