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Pactamycin Binding Site on Archaebacterial and Eukaryotic Ribosomes†

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ABSTRACT: The presence of a photoreactive acetophenone group in the protein synthesis inhibitor pactamycin and the possibility of obtaining active iodinated derivatives that retain full biological activity allow the antibiotic binding site on *Saccharomyces cerevisiae* and archaebacterium *Sulfolobus solfataricus* ribosomes to be photoaffinity labeled. Four major labeled proteins have been identified in the yeast ribosome, i.e., YS10, YS18, YS21/24, and YS30, while proteins AL1a, AS10/L8, AS18/20, and AS21/22 appeared as radioactive spots in *S. solfataricus*. There seems to be a correlation between some of the proteins labeled in yeast and those previously reported in *Escherichia coli* [Tejedor, F., Amils, R., & Ballesta, J. P. G. (1985) *Biochemistry 24*, 3667–3672], indicating that the pactamycin binding sites of both species, which are in the small subunit close to the initiation factors and mRNA binding sites, must have similar characteristics.

Although the basic steps of protein synthesis have been maintained throughout evolution, significant structural differences prevent a direct extrapolation of the data from prokaryotic to eukaryotic ribosomes.

The situation is further complicated by the description of the archaebacterial kingdom, comprised of unicellular organisms, thus far classified as bacteria, with peculiar properties (Woese, 1982). From the point of view of protein synthesis, archaebacteria have characteristics, such as ribosome structure (Lake et al., 1984) and sensitivity to antibiotics (Elhardt & Böck, 1982; Cammarano et al., 1985), differentiating them from eukaryotic and prokaryotic cells.

The mechanism of protein synthesis and the structure of the different components involved (ribosomes, tRNAs, soluble factors, etc.) are starting to be reasonably well understood in eubacteria. In spite of recent advances, data are relatively scarce for eukaryotic cells (Bielka, 1982) and almost nonexistent for archaebacteria. It would therefore be of great interest to establish correlations among the three systems that would facilitate the use of the wealth of data available on eubacteria to understand the process in the other two systems.

Antibiotics have been used as powerful tools in the investigation of the protein synthesis mechanism and the structure of its components, especially the ribosomes. The characterization of the antibiotic's target in the protein synthesis machinery allows the correlation of its components with the functions inhibited by the drug. Ribosomal components involved in peptide bond formation and interaction with elongation factors have been located by identifying the binding sites of antibiotics that inhibit these activities, such as chloramphenicol (Pongs & Messer, 1976), macrolides (Tejedor & Ballesta, 1985), puromycin (Nicholson et al., 1982), thiostrepton (Thompson et al., 1979), and tetracycline (Goldman et al., 1983).

Antibiotics are known to be specific inhibitors of either eukaryotic or eubacterial protein synthesis (Vâzquez, 1979), although some of them are able to affect both systems. The use of the last class of drugs is especially suited for comparative studies since the identification of their respective targets allows a correlation between their components in the different cell types to be established.

Pactamycin, a representative of these wide-spectrum antibiotics, was initially reported as an antitumor drug that also inhibited the growth of bacterial cells (Goldberg, 1974). It has been suggested that pactamycin inhibits the initiation step of protein synthesis by interacting with the small ribosomal subunit (Goldberg, 1974). The identification of the components of the pactamycin binding site in ribosomes from different species may thus allow the correlation of the ribosomal components involved in the initiation of protein synthesis in different systems.

The pactamycin binding site can be easily studied by affinity-labeling methods, since its molecule contains an acetophenone group that is photoreactive as well as a phenolic group that is susceptible to radioactive labeling by iodination (Figure

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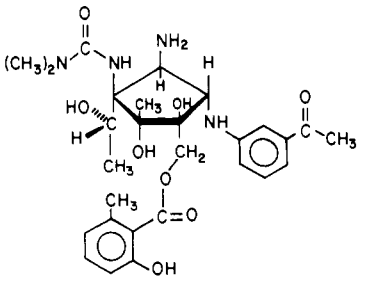

FIGURE 1: Chemical structure of pactamycin according to Weller and Rinehart (1978).

1). These advantages have already been exploited in our laboratory to investigate the structure of the pactamycin binding site on the *Escherichia coli* ribosome (Tejedor et al., 1985).

In this paper we present results that extend this study to ribosomes of a eukaryotic cell, *Saccharomyces cerevisae*, and an archaebacterial species, *Sulfolobus solfataricus*. The data obtained allowed us to identify ribosomal proteins involved in the initiation steps of protein synthesis in the three species. It may be possible to locate the positions of the corresponding proteins in the eukaryotic and archaebacterial ribosomal models by comparing them with data obtained from *E. coli* (Tejedor et al., 1985).

Materials and Methods

Ribosomes. Cells from *S. cerevisiae* Y166 grown in a yeast extract–peptone–glucose medium up to late exponential phase were ground with sea sand and ribosomes prepared according to standard procedures (Sanchez Madrid et al., 1981). Ribosomal subunits were prepared by zonal centrifugation on sucrose gradients. *S. solfataricus* was grown at 85 °C up to exponential phase (A_{600} = 0.6) in an acidic medium (pH 3.0) containing amino acids, yeast extract, and salts (Cammarano et al., 1982). Cells were disrupted with alumina and ribosomes were obtained as indicated elsewhere (Cammarano et al., 1982).

Iodination of Pactamycin. Pactamycin was iodinated by use of chloramine T and avoidance of direct contact of the oxidant compound with the drug as described previously (Tejedor & Ballesta, 1982). After iodination the modified drug was purified by chromatography on Sephadex LH20 followed by TLC[1] on silica gel (Tejedor et al., 1985).

Activity Tests. Inhibition of protein synthesis was tested by using an S-30 cell-free system from rabbit reticulocytes (Pelham & Jackson, 1976).

Binding of [^{125}I]iodopactamycin to ribosomes was assayed by filtration through nitrocellulose filters and by centrifugation through either 5–20% or 15–30% sucrose gradients as described (Tejedor et al., 1985).

Photoincorporation of Iodopactamycin to Ribosomes. Ribosomes (1–1.2 μM), extensively dialyzed against 10 mM sodium borate, pH 7.0, 100 mM KCl, and 10 mM $MgCl_2$ and incubated in the dark at 0 °C for 20 min with 0.15 μM [^{125}I]iodopactamycin, were irradiated with a 125-W medium-pressure mercury lamp using a borosilicate filter to cut out radiation below 300 nm. The irradiation was performed in a refrigerated bath to keep the temperature of the sample below 10 °C.

Incorporation of [^{125}I]iodopactamycin was tested by precipitation with 10% trichloroacetic acid at 0 °C followed by rapid filtration through glass fiber filters.

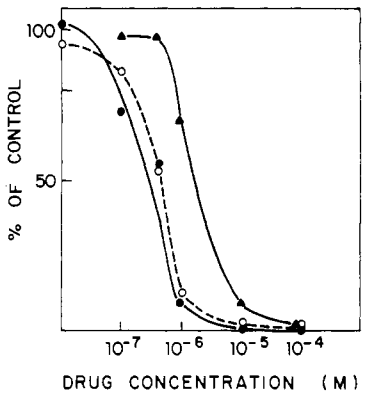

FIGURE 2: Inhibition of a reticulocyte S-30 cell-free system by pactamycin (○), iodopactamycin A (●), and iodopactamycin B (▲).

Analysis of Ribosomal Proteins. RNA and proteins were separated by urea–LiCl treatment (Traub et al., 1971). Proteins were prepared for electrophoresis by treating the ribosomes with RNase A and RNase T1 (León Ribera et al., 1980). After RNase treatment, proteins were precipitated with 3 volumes of acetone at −25 °C and then dissolved in sample buffer for two-dimensional gel electrophoresis. Proteins from yeast were separated by the standard method (Kaltschmidt & Wittman, 1970) and proteins of *S. solfataricus* according to Londei et al. (1982).

After electrophoresis, gels stained with 0.4% Coomassie blue in 50% ethanol were kept in 60% acetone at 0 °C for 2 h. Destaining was carried out at room temperature in 25% ethanol overnight. Slabs were dried and exposed on Kodak X-Omat S radiographic plates.

Quantification of spots was performed by using a Photomation P-1700 densitometer from Optronics connected to a Digital Pdp-11 computer.

Protein nomenclature for *S. cerevisiae* and *S. solfataricus* was as proposed by Kruiswijk and Planta (1974) and Londei et al. (1982), respectively. In order to differentiate proteins from different sources we have placed before the numbers a Y for yeast proteins, an A for archaebacterial, and an E for *E. coli*.

Results

Biological Activity of Iodopactamycin. Pactamycin is susceptible to iodination, probably through the phenol group present in its molecule (Figure 1). Two major derivatives, iodopactamycins A and B, which can be easily purified by thin-layer chromatography, are isolated (Tejedor et al., 1985). Both pactamycin derivatives are biologically active when tested in in vivo and in vitro systems although, as shown in Figure 2 for a reticulocyte S-30 protein-synthesizing assay, iodopactamycin B is considerably less active than the unmodified drug, while iodopactamycin A is as active as pactamycin. All consecutive experiments were performed with iodopactamycin A.

The binding of [^{125}I]iodopactamycin A to ribosomes from different sources was tested by sucrose gradient centrifugation. Binding to the small subunit and to the whole ribosomes from *S. cerevisiae* and *E. coli* takes place (Figure 3). In the case of the archebacterium *S. solfataricus*, centrifugation through sucrose gradients of the ribosomes under different ionic conditions always results in the total dissociation of the particles. Therefore, only binding to the 30S subunit could be detected by this technique (Figure 3).

Low concentrations of the unmodified drug compete with [^{125}I]iodopactamycin for binding to ribosomes. As shown in

[1] Abbreviations: TCA, trichloroacetic acid; TLC, thin-layer chromatography.

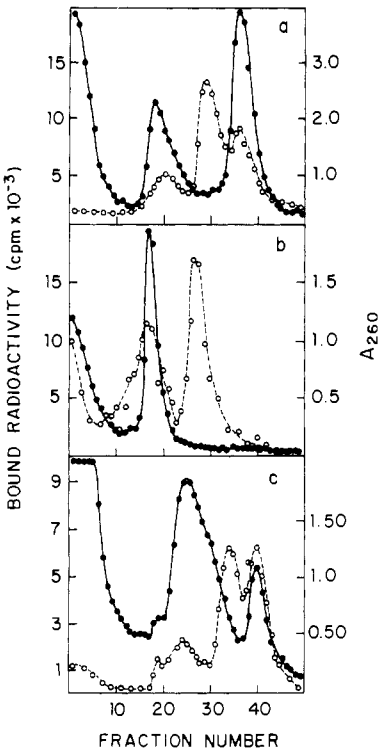

FIGURE 3: Binding of [^{125}I]iodopactamycin A to ribosomes of *E. coli* (a), *S. solfataricus* (b), and *S. cerevisiae* (c). Binding was performed with 0.5–0.75 μM ribosomes and 1.5-fold excess of antibiotic. After incubation the samples were centrifuged through 5–20% (*E. coli*) or 15–30% (*S. cerevisiae* and *S. solfataricus*) sucrose gradients, which afterward were fractionated, and the optical density (○) and radioactivity (●) were measured.

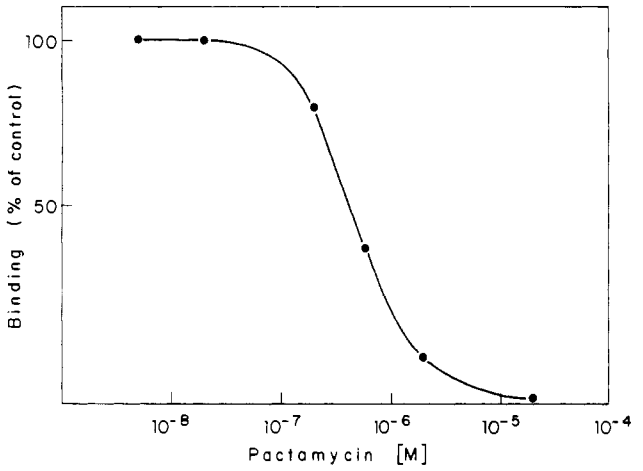

FIGURE 4: Competition of [^{125}I]iodopactamycin binding by pactamycin. Ribosomes from *S. cerevisiae* (0.5 μM) were incubated with 1 μM [^{125}I]iodopactamycin in the presence of increasing concentrations of untreated pactamycin, and the binding was tested by filtration through nitrocellulose. One hundred percent binding corresponds to 0.5 mol of drug/mol of ribosome.

Figure 4, 50% inhibition of the binding is obtained with a concentration of cold pactamycin similar to that of the radioactive drug. These results strongly indicate that both compounds bind with similar affinity to identical ribosomal sites.

Covalent Binding of Iodopactamycin. When ribosomes from yeast are irradiated in the presence of [^{125}I]iodopactamycin A, under the conditions indicated under Materials and Methods, the radioactivity remains bound to the particles following precipitation with TCA (Figure 5). Similar results were obtained in the case of eubacterial (Tejedor et al., 1985)

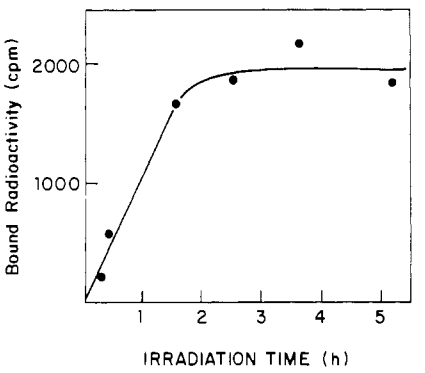

FIGURE 5: Covalent binding of [^{125}I]iodopactamycin to *S. cerevisiae* ribosomes. Yeast ribosomes and [^{125}I]iodopactamycin were incubated in the conditions described in Figure 4 and then irradiated as described previously. Twenty-five-microliter aliquots were taken out, precipitated with 5% trichloroacetic acid, and filtered through glass fiber filters.

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

	radioactivity in	
	small subunit (cpm)	whole ribosome (cpm)
rRNA	4930	4079
protein	3522	5860
ratio RNA/protein	1.4	0.7

[a] One hundred microliters of either small subunits or whole ribosomes of *S. cerevisae* at 0.5 μM was incubated with [^{125}I]iodopactamycin and irradiated under the conditions described under Materials and Methods. Ribosomal proteins and RNA were separated by the LiCl–urea method and reprecipitated twice with acetone and ethanol, respectively, and the radioactivity associated with each fraction was measured.

and archaebacterial ribosomes (not shown).

Radioactivity covalently associated to the ribosomes was found in the RNA and protein moieties of the particles (Table I), although the ratio of the amount incorporated into the two fractions changes when binding and irradiation are carried out with either whole ribosomes or small subunits. These results indicate a preferential labeling of proteins in the ribosome and of RNA in the subunits, suggesting a change in the binding site conformation upon subunit association.

Identification of the Labeled Proteins. Proteins extracted from yeast and *S. solfataricus* ribosomes were separated by two-dimensional gel electrophoresis, and the labeled spots were detected by autoradiography. Figure 6 shows the stained gels and the corresponding autoradiograms obtained from yeast and *S. solfataricus* ribosomes. Very little radioactivity is detected along the first-dimension gels of the basic and acidic proteins, suggesting that all the material entered the second-dimension slab. The radioactivity in the origin of the acidic protein first-dimension gel must correspond to some basic proteins that have penetrated the top layers of the gel; part of them can afterward move into the second dimension, giving the radioactive spots found in the region between the acidic part and the basic part of the slab. After modification, the position of the labeled proteins is modified, moving toward the cathode more slowly than the unlabeled spots. In 40S subunits from *S. cerevisiae*, proteins YS21/24 and YS30 are strongly labeled, proteins YS10 and YS27 are moderately labeled, and some radioactivity is found in YS18 and YS26. In the case of *S. solfataricus*, labeling was performed with whole 70S ribosomes and the detected radioactivity was associated to spots that contained proteins AS21/22, AS8/20, AS10/L20, and AL1a.

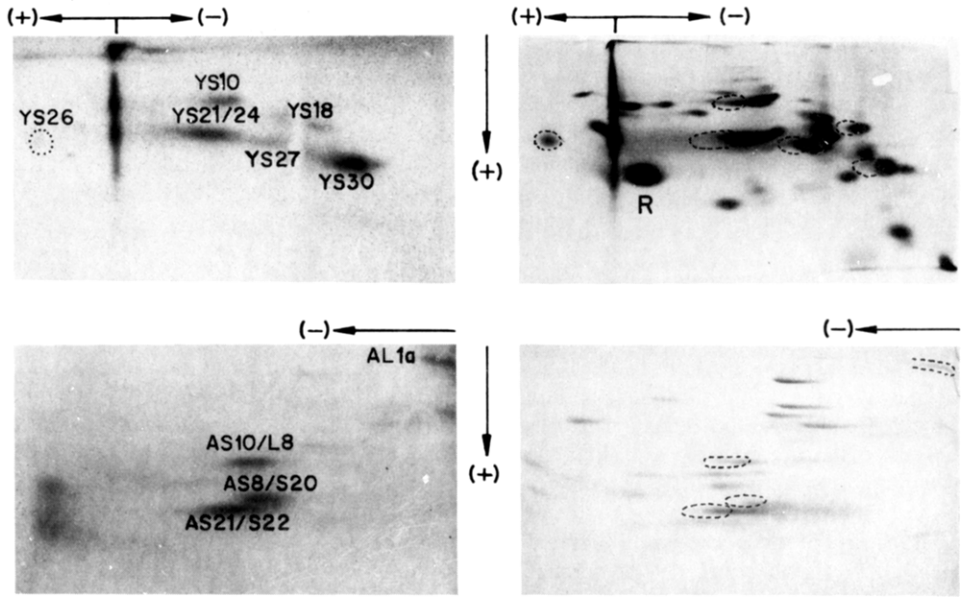

FIGURE 6: Two-dimensional gel electrophoresis (right panels) and the corresponding autoradiograms (left panels) of proteins from [^{125}I]-iodopactamycin-labeled *S. cerevisae* 40S subunits (upper panels) and *S. solfataricus* ribosomes (lower panels). Spots in the left side of the *S. solfataricus* autoradiogram do not correspond to any stained protein. R indicates the position of RNase used for protein preparation.

Table II: Quantification of Ribosomal Protein Labeling by [^{125}I]Iodopactamycin

	irradiation performed in the presence of 10^{-6} M [^{125}I]iodopactamycin and					
	no unlabeled drug (control)		2.5×10^{-6} M unlabeled drug		10^{-5} M protein, unlabeled drug	
protein	OD[a]	%	OD[a]	%	OD[a]	%
YS10	201.8	100	40.0	19.8	4.1	2.1
YS18	25.6	100	25.7	100	9.5	37
YS21/24	656.9	100	191.0	34.8	84.9	12.9
YS26	22.7	100	0	0	0	0
YS27	148.1	100	52.8	35.6	36.4	24.5
YS30	689.9	100	238.8	34.6	68.0	9.8
total protein	1745	100	548.3	31.4	202.9	11.6

[a] Optical density in arbitrary units.

Specificity of the Protein Labeling. In order to test whether the labeling of the individual proteins was due to a specific interaction of the drug, irradiation of the yeast ribosomes in the presence of [^{125}I]iodopactamycin and increasing concentrations of nonradioactive antibiotic was carried out. The proteins were separated by two-dimensional gel electrophoresis, and the intensity of the spots in the corresponding autoradiograms was quantified by computerized densitometry. Table II shows that in the presence of a 2.5-fold excess of unlabeled pactomycin most proteins incorporate about 30% of the radioactivity present in the controls while a 10-fold excess reduces the incorporation to 10% of the controls. These results agree with the competition experiment shown in Figure 4 and confirm that the labeling of the individual proteins must be due to a specific interaction of the drug with the ribosome. Protein YS18, however, seems to be an exception since its labeling is either unaffected or only reduced to 40% by the 2.5- and 10-fold excesses of unlabeled drug, respectively, suggesting less specificity in its labeling.

Discussion

The results obtained by the pactamycin affinity labeling of *E. coli* ribosomes indicate that the binding site of the drug is located on the basis of the cleft between the platform and the body of the small ribosomal subunit (Tejedor et al., 1985). This is a region formed by proteins ES18 ES4, ES2, and ES21, among others, where mRNA and the initiation factors seem to interact with the ribosome, in agreement with the proposed mode of action of the drug (Goldberg, 1974; Vázquez, 1979). It is reasonable to assume that the position of the binding sites on ribosomes from other species is the same as in *E. coli*.

Little is known about the three-dimensional structure of the yeast ribosomes, but the scarce data available indicate the existence of structures analogous to those found in eubacterial particles. The small subunit seems to have a head and a body with a lateral protuberance or platform (Lake et al., 1984), and by analogy with *E. coli*, the pactamycin-labeled proteins, YS10, YS21/24, and YS30 in yeast and probably AS8/20, AS10, and AS21/22 in *S. solfataricus*, must be located in the body near the base of the lateral platform, a ribosomal domain where interaction with mRNA and the initiation factors probably takes place.

The ribosome structure and the functional implications of ribosomal proteins have been studied in more detail in rat liver (Bielka, 1982). Unfortunately, in most cases an equivalence has not been established between ribosomal proteins from different sources, and therefore it is difficult to extend our results to the higher cell's particles. Nevertheless, some correlations can be established that may help to gain some insight into the yeast ribosome. Protein YS10 has been reported to be the counterpart of mammalian protein S6 mainly on the basis of its electrophoretic mobility and of its capacity to be phosphorylated under certain growth conditions (Zinker & Warner, 1976). Mammalian protein S6 has been implicated in protein synthesis initiation and in the binding of mRNA to ribosomes by several criteria such as cross-linking to poly(uridylic acid) (Terao & Ogata, 1979), studies of the role of ribosome phosphorylation on protein synthesis activity (Burkhard & Traugh, 1983), and inhibition by specific antibodies (Bommer et al., 1980). The highly specific labeling of yeast YS10 by pactamycin, which specifically inhibits the initiation of protein synthesis (Goldberg, 1974), is in agreement with these data and confirms the role of this polypeptide in the initiation steps of the synthetic process. In *E. coli*, protein ES4, whose electrophoretic mobility is similar to those of mammalian S6 and yeast YS10 and which has been related to the initiation of protein synthesis and binding of mRNA

[see Liljas (1982) for a review], is also labeled by pactamycin (Tejedor et al., 1985). Although conclusions are still speculative, these three proteins may have similar functional and structural roles in their respective ribosomes; if this is so, all of the data on bacterial ES4 should be taken into account when the equivalent proteins in higher cells are studied. A comparison of the primary, secondary, and tertiary structures of the three polypeptides, when available, would be highly interesting.

The proteins labeled by pactamycin in the *S. solfataricus* ribosome must be located, by analogy with the results in the other species, in a ribosomal region involved in the interaction with initiation factors and mRNA. The few electron microscopy studies of archaebacterial ribosomes available to date also indicate the existence of a head, a body, and a platform in the small subunit (Lake et al., 1984), suggesting that the pactamycin binding site in archaebacterial particles is probably in a similar position as in eubacterial and eukaryotic particles. Unique physicochemical features of archaebacterial ribosomal proteins (Schmid & Böck, 1982; Londei et al., 1982) together with the different electrophoretic systems prevent a direct individual correlation between the labeled proteins in *S. solfataricus* and the other two species to be established.

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Registry No. Pactamycin, 23668-11-3.

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