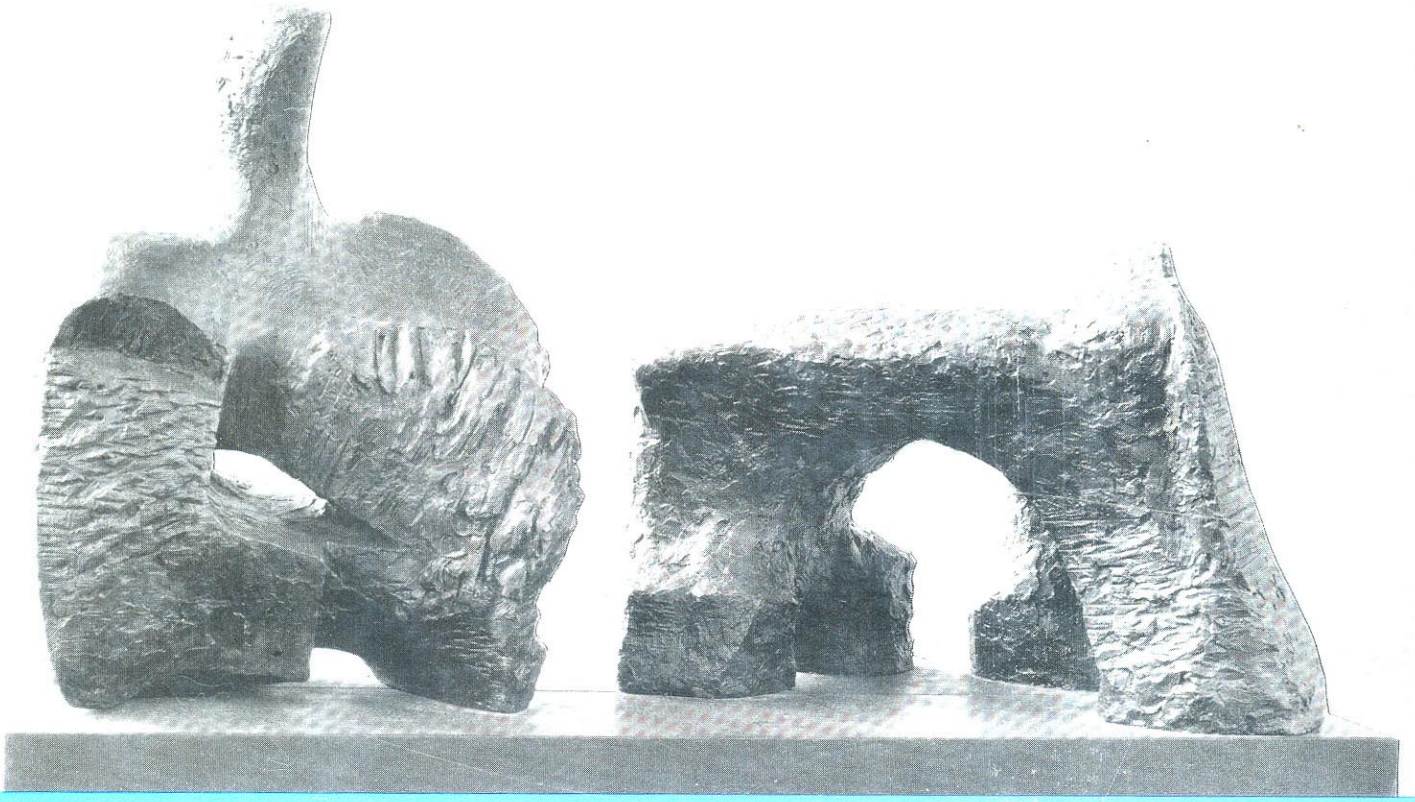
THE RIBOSOME



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Chapter 8

Crystallography and Image Reconstructions of Ribosomes

A. YONATH, W. BENNETT, S. WEINSTEIN, and H. G. WITTMANN

In this chapter, we describe results of the application of X-ray crystallography and image reconstruction to intact ribosomal particles. Our initial attempts at crystallization, the first and most crucial step in these studies, were summarized previously (Yonath et al., 1986c). Here we emphasize recent advances in the crystallographic work and future prospects.

CRYSTALLIZATION AND X-RAY STRUCTURE ANALYSIS OF RIBOSOMAL PARTICLES

Earlier reports from our laboratories have concentrated on our efforts initially to obtain and later to improve the quality of diffraction from crystals of native ribosomal particles. Thanks largely to improvements in the techniques used for growing crystals (Yonath et al., 1988a; Yonath and Wittmann, 1989a) and for collecting crystallographic data from shock-cooled crystals (Hope et al., 1989), we have recently begun to obtain single-crystal diffraction pattern information approaching atomic resolution from some of the crystal forms described earlier. Although our efforts to improve the existing crystal forms continue, these recent successes have encouraged us to devote more effort to the problem of determining initial phases and to the related problem of obtaining crystals of functionally interesting complexes of ribosomal particles with other components of protein synthesis.

Table 1 summarizes the crystals of ribosomal particles grown in our laboratories to date. It is clear from the table that we have been able to obtain crystals from a mutant and a number of chemically modified particles, in addition to native particles. One can also see that some of our efforts to cocrystallize ribosomal particles with other components necessary for protein biosynthesis have already succeeded, in particular several involving complexes with tRNA. Two factors seem to be important in the crystallization of ribosomal particles and their complexes. One is the use of functionally active preparations. We consistently obtain the best crystals from the most active preparations. In addition, we routinely dissolve samples of crystals to verify that the crystalline particles have retained their activity; in all cases, the resolubilized material retains its integrity and biological activity, even when it has been in the crystalline form for periods of several months. A second factor that may be of importance is that the crystals most suitable for crystallographic study are of ribosomal particles from thermophilic or halophilic bacteria; presumably the ribosomes from these organisms are more stable than those from eubacteria during isolation and crystallization.

The column "Resolution" in Table 1 refers to the highest resolution for which diffraction spots could be consistently observed on films. Since there remains considerable variability in the resolution of the diffraction data obtained from different crystals, even among crystals from the same batch, we believe that the best resolution listed in the table is only a temporary upper limit that is likely to improve as we continue to identify and control the sources of the current variability. This is particularly true of the best-diffracting crystals to date, those of the 50S subunit from *Halobacterium marismortui*; these crystals are thin plates that are evidently very susceptible to the mechanical stresses of shock cooling, and

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Table 1. Characterized three-dimensional crystals of ribosomal particles

Source	Crystal form[a]	Cell dimensions (Å) determined by:		Resolution (Å)	Comments[b]
		Electron microscopy	X-ray crystallography		
70S *E. coli*	A	340 × 340 × 590; $P6_3$			
T. thermophilus					
70S	M		524 × 524 × 306; $P4_12_12$	19	
30S	M		407 × 407 × 170; $P42_12$	7.3	N, H
50S *H. marismortui*	1, P	310 × 350; 105°			
	2, P	148 × 186; 95°	147 × 181; 97°	13	
	3,[c] P	170 × 180; 75°	210 × 300 × 581; $C222_1$	4.5	N, H
50S *B. stearothermophilus*	1, A	130 × 254; 95°			
	2, A	156 × 288; 97°			
	3, A	260 × 288; 105°			
	4, A	405 × 405 × 256; 120°			
	5, A	213 × 235 × 315; 120°			
	6,[c,d] A	330 × 670 × 850; 90°	360 × 680 × 920; $P2_12_12$	18	N
	7,[c,d] P		308 × 562 × 395; 114° C2	11	N, H

[a] Crystals were grown by vapor diffusion from low-molecular-weight alcohols (A), MPD (M), or polyethylene glycol (P).
[b] Crystallographic data were collected from native (N) and derivatized (H) crystals.
[c] Same form and parameters for crystals of a complex of 50S subunits plus tRNA and a segment (18- to 20-mers) of a nascent polypeptide chain.
[d] Same form and parameters for crystals of large ribosomal subunits of a mutant (deficient in L11 protein) of the same source.

we anticipate that the intrinsic order of the crystals is even better than the resolution of 4.5 Å (0.45 nm) listed in the table, which already begins to approach atomic resolution (Fig. 1).

Despite extensive attempts to crystallize small ribosomal subunits, crystals of these particles were obtained only recently (Glotz et al., 1987; Trakhanov et al., 1987; Yusupov et al., 1988; Yonath et al., 1988b), long after the crystallization procedures for several crystal forms containing 50S subunits were well established. The difficulty we and others have encountered in obtaining crystals of 30S subunits is probably related to the relative instability of these particles compared with the 50S subunits from the same organism. Evidence for this point of view has recently been obtained in our laboratories by exposing 70S ribosomes from *Echerichia coli* to a preparation of proteolytic enzymes from *Aspergillus oryzae*. Large variations in the resistance of the two subunits were observed; the 50S subunits remained intact, whereas the 30S subunit completely disintegrated (U. Evers and H. S. Gewitz, unpublished data).

The first microcrystals of 70S ribosomes from *E. coli* were obtained 7 years ago (Wittmann et al., 1982). These crystals were well ordered but too small for crystallographic analysis. More recently, we and

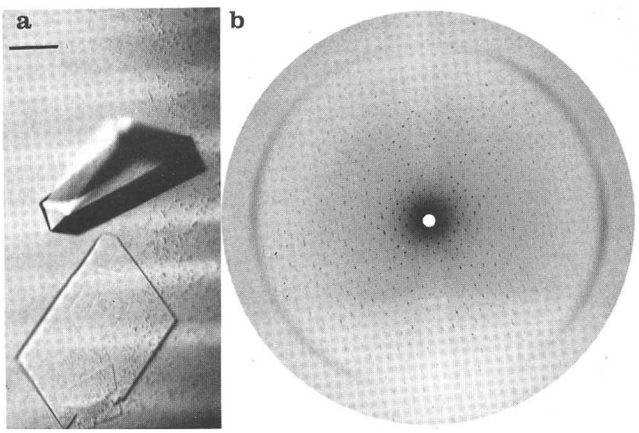

Figure 1. (a) Crystals of 50S subunits of *H. marismortui* grown as described by Makowski et al. (1987). Bar, 0.2 mm. (b) A 1° rotation pattern (recorded on film) of a crystal similar to the one shown in panel a but soaked in a solution containing the components used for its growth and 18% ethylene glycol. The pattern was obtained after 27 h of irradiation at −180°C with a synchrotron X-ray beam (X11 port at EMBL/DESY). Wave length, 1.488 Å; exposure time, 7 min; crystal-to-film distance, 205 mm; resolution, 4.5 Å.

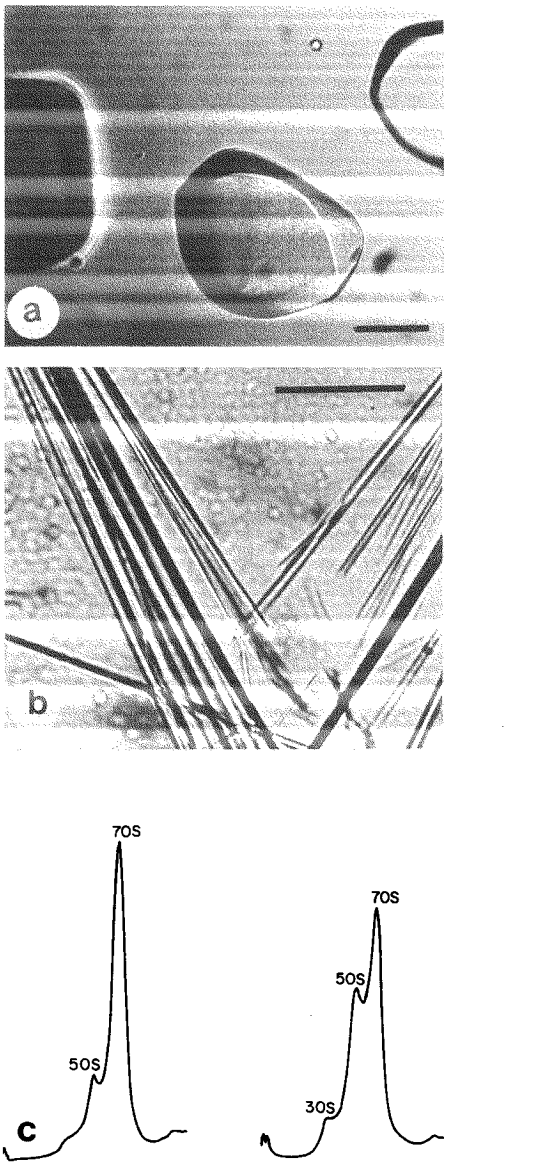

Figure 2. Crystals of 70S ribosomes from *T. thermophilus* (a) and *B. stearothermophilus* (b). Bar, 0.2 mm. (c) Sucrose gradient profiles of the material used for crystallization (left) and of dissolved crystals of *B. stearothermophilus* (right).

others have been able to grow crystals of 70S particles from two thermophilic eubacteria: *Bacillus stearothermophilus* (wild type as well as a mutant) and *Thermus thermophilus* (Glotz et al., 1987; Trakhanov et al., 1987; Berkovitch-Yellin et al., in press [a]). The 70S particles from both sources produce crystals of intermediate size (compared with crystals of other ribosomal particles; Fig. 2), but the crystals consistently diffract to only low resolution: 35 Å for the 70S particles from *B. stearothermophilus* and 19 Å for those from *T. thermophilus*. Given the correlation that we have observed between the activities of samples of 30S or 50S subunits and the quality of the crystals obtained from them, we believe that the poor internal order of the crystals of 70S particles is due to the conformational and functional heterogeneity of the tight couples used for crystallization in our laboratories.

We anticipated that 70S ribosomes obtained by association of purified 50S and 30S subunits will provide a more homogeneous and defined population for obtaining 70S particles. Crystallization experiments have been prepared with several different preparations of high activity but have so far failed to yield crystals. A particularly interesting result was obtained in a similar experiment with highly active hybrids of 50S subunits from *B. stearothermophilus* and 30S subunits from *E. coli*. The crystallization of this hybrid particle was attempted in solutions rich in Mg^{2+}, using a crystallization medium similar to the buffer system used for in vitro poly(U)-programmed polyphenylalanine synthesis. Although large, well-ordered crystals were obtained, sucrose gradients of dissolved crystalline material showed that these crystals contained only 50S particles, despite the presence of 30S subunits in the crystallization mixture (Fig. 3). This result is consistent with our previous observations that large ribosomal subunits crystallize readily under a variety of conditions (Table 1; Yonath et al., 1980; Yonath et al., 1983; Yonath et al., 1984; Yonath et al., 1986a; Yonath et al., 1986b; Yonath et al., 1986c; Müssig et al., 1989). The fact that the 50S subunits crystallized in the presence of heterologous 30S subunits indicates that the interparticle contacts between large subunits in the crystal are stronger than the affinity between the large and small subunits in this hybrid 70S particle, even though the hybrid ribosomes are fully active in vitro. A similar observation has been made for packed two-dimensional sheets of eucaryotic 80S ribosomes. These sheets could be depleted of small subunits and still maintain their packing integrity as a lattice of large subunits (Kühlbrandt and Unwin, 1982).

The crystallization conditions for most of the crystal forms listed in Table 1 are sufficiently refined (and our preparative techniques sufficiently reproducible) that we can obtain crystals from virtually all preparations of active particles. However, some variability in the ribosome preparation evidently still exists, since the exact conditions for the growth of well-ordered and large crystals still must be refined for each ribosomal preparation (Yonath and Wittmann, 1989a). It is interesting that for particles which can be crystallized in more than one form, a preparation that yields large, well-shaped crystals of one crystal form will generally also crystallize well in the other forms. For example, preparations of 50S subunits from *B. stearothermophilus* that produce

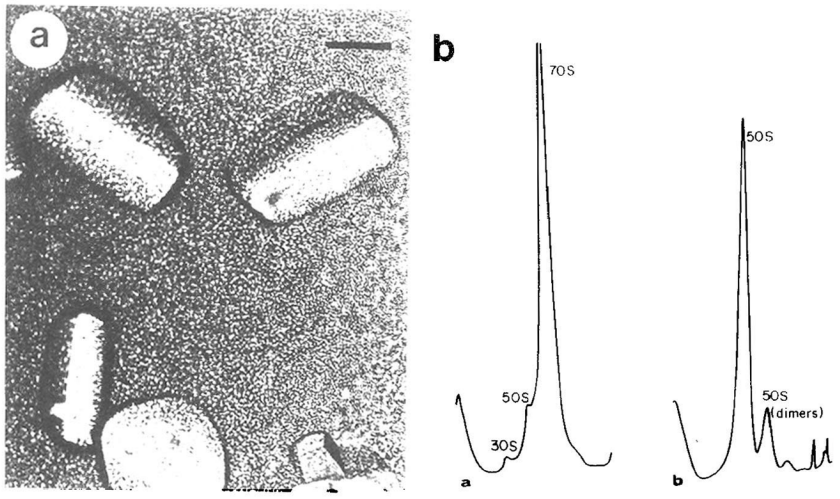

Figure 3. (a) Crystals obtained from an attempt to crystallize a hybrid of 50S from *B. stearothermophilus* and 30S from *E. coli*. Bar, 0.2 mm. (b) Sucrose gradient profile of the material used for crystallization (left) and of the dissolved crystals (right).

good crystals when crystallized from alcohol solutions (form 6 in Table 1) also yield high-quality crystals when polyethylene glycol is used as the crystallizing agent (form 7). Thus, the basic factors governing the ease of crystallization and the yield of large, well-formed ribosomal crystals seem to be related more to the quality of the preparation of the ribosomal particles than to the choice of the crystallization agent.

B. stearothermophilus is the only source from which crystals of all ribosomal particles (70S, 50S, and 30S, including mutated and chemically modified particles) can be grown. The ribosomes and ribosomal subunits of *B. stearothermophilus* have been well characterized by chemical, physical, and immunological methods and thus are particularly attractive for structural study. Furthermore, crystals of the ribosomal particles from *B. stearothermophilus* have been obtained from solutions that resemble the natural environment of the ribosome in the cell, except for the addition of a small amount of polyethylene glycol. For these reasons, we continue our studies of ribosomal crystals from this organism, despite the relatively poor resolution of the diffraction patterns that we have obtained from these crystals to date (Table 1).

The diffracting power of crystalline ribosomal particles is so weak that virtually all of the crystallographic studies have been performed with synchrotron radiation (Bartels et al., 1988). The extremely intense X rays available at synchrotron installations are generated as a by-product of accelerators originally designed for high-energy-particle experiments. However, at temperatures ranging from room temperature to about 4°C, the radiation damage suffered by all crystals of ribosomal particles in an intense synchrotron beam is so rapid and so severe that all reflections beyond about 18 Å resolution decay within a few minutes, a period shorter than the time required to obtain a single X-ray photograph. In our early diffraction studies of crystals of ribosomal particles, which were performed at temperatures above 4°C, the extreme sensitivity of the crystals to radiation damage led us to conclude (incorrectly, as we discovered later) that the diffraction was limited to 15 to 18 Å resolution (Yonath et al., 1984; Yonath et al., 1986a; Yonath et al., 1986b; Yonath et al., 1986c). Even when the problem of radiation damage was evident, the diffraction that we could observe at these temperatures could be seen only in the first X-ray photograph from a crystal. Thus, to measure the diffraction data even to this resolution, a new crystal had to be used for each photograph, and precise alignment of the crystals was impossible. In addition, the exposure of each crystal had to be kept as short as possible to minimize the effect of radiation damage. In a typical attempt to obtain a complete diffraction data set under these conditions, more than 260 individual crystals were used. Typically, however, the combination of randomly oriented crystals and short exposures yielded only partial diffraction data sets which did not contain even a single fully recorded reflection, making evaluation of the data difficult at best.

Since the time of these early diffraction studies, we have been able to overcome the problem of radiation sensitivity for all of the crystals of ribosomal particles by shock freezing the crystals to cryogenic temperatures (for our work, the boiling temperature of liquid nitrogen, about −180°C) be-

fore the diffraction experiment. It is generally believed that a major component of the damage to biological samples by ionizing radiation is caused by the diffusion of free radicals, which are produced throughout the region of the sample that is irradiated, including the solvent regions of macromolecular crystals. Small-molecule crystallographers have routinely performed data collection at cryogenic temperatures to protect radiation-sensitive samples from X-ray damage for years, but at the outset of our attempts to apply this approach to ribosomal crystals, the use of low temperatures with macromolecular crystals had generally been less successful, often because the crystals could not be transferred to the cryosolvents thought at the time to be necessary to prevent the solvent in the crystals from freezing. We were able to use the then novel technique of shock freezing in the absence of cryoprotectants to crystals of ribosomal particles (Hope et al., 1989); with this approach, the formation of ice crystals is prevented by lowering the temperature so rapidly that the solvent solidifies as an amorphous glass before crystals can form. It has proven possible to apply the shock-freezing technique to all crystals of ribosomal particles that we have studied so far, with the result that the frozen crystals, if maintained at cryogenic temperatures, suffer no observable radiation damage over the time span needed for collecting several complete diffraction data sets, which allows even the weakest diffraction data available to be measured. We have also recently developed techniques for storing shock-frozen crystals for long periods in solid propane, which solidifies as a glass at a temperature a few degrees above the boiling point of nitrogen. These techniques can also be applied to crystals that have been irradiated, allowing a diffraction experiment to be interrupted and resumed at a later date.

SPECIFIC AND QUANTITATIVE LABELING OF RIBOSOMAL PARTICLES

Determination of the three-dimensional structure of a crystalline compound by X-ray crystallography involves a Fourier summation of the reflections present in the diffraction pattern. Each reflection is a wave characterized by its direction, intensity, and phase. What keeps this summation from being a trivial computational problem is the fact that only the direction and amplitude of a reflection can be measured, whereas the phase cannot be directly determined. For macromolecular crystals, two techniques are commonly used to determine the phases. If an approximate model is available or if the unknown macromolecule is closely related to one whose structure is known, the known structure can often be used to provide preliminary phase information for the unknown molecule with the aid of various search procedures that allow the known structure to be positioned in the crystal lattice of the unknown molecule; this approach is known as molecular replacement. If the crystalline macromolecule is not related to one whose structure is known, its phases are most often determined by the multiple isomorphous replacement (MIR) method. Phase determination by MIR involves the preparation of at least two chemical modifications of the unknown molecule, usually the addition of one or a few electron-dense atoms or groups of atoms to the structure. The modification must be large enough to cause measurable changes in the diffraction pattern of the unknown molecule but not so extensive as to change the structure of the molecule or its crystal lattice. If these conditions are met and the number of added groups is small, the location of the added groups can usually be deduced from the changes in the diffraction pattern, which in turn allows the phases for the unmodified structure to be determined.

Because ribosomal particles are much larger than any macromolecular complex solved by X-ray crystallography to date, one can anticipate that it will be difficult to fulfill all of the conditions of an ideal isomorphous derivative simultaneously; for example, it could be difficult to produce measurable changes in the diffraction pattern of a ribosomal particle with only a few scattering groups. To address at least the foreseeable difficulties of phase determination of such large structures, we have adopted a strategy that combines elements of both the molecular replacement and MIR methods. We have previously reported low-resolution structures derived by image reconstruction from electron micrographs of two-dimensional, ordered arrays of ribosomal particles (Yonath et al., 1987a; Yonath et al., 1987b; Arad et al., 1987b; Yonath and Wittmann, 1989b). These reconstructions are being used as approximate models of the crystalline ribosomal particles for molecular replacement. It is our hope that the low-resolution phases obtained by this approach will be useful for locating additional scattering groups in isomorphous derivatives, perhaps even large numbers of added groups, which will provide higher-resolution MIR phases.

For determination of the phases of proteins with molecular weights of up to about 50,000, the added group of a useful isomorphous derivative typically consists of one or two heavy-metal atoms. An ideal added group for the ribosomal particles would consist of a compact cluster of a proportionately larger number of heavy atoms. Clusters with a core of

several heavy-metal atoms linked directly to one another have been synthesized (Jahn, 1989a, 1989b) and are attractive candidates for isomorphous substitutions of ribosomal particles. We are using two such clusters: an undecagold cluster with a total molecular weight of 6,200 and a tetrairidium cluster with a molecular weight of 2,300. As the core of the undecagold cluster is about 8.2 Å in diameter, it can be treated as a single scattering group at low to medium resolution. The tetrairidium cluster has a core diameter of about 5 Å and can thus be treated as a single heavy atom to somewhat higher resolution. Although these clusters have not been used previously for phasing by protein crystallographers, they seem to be ideally suited for crystallographic analysis of ribosomal particles.

In forming isomorphous derivatives of ribosomal particles with these clusters, we are attempting to take advantage of the extensive knowledge of the biochemistry, biophysics, and genetics of the ribosome to prepare singly substituted derivatives that should produce easily interpretable changes in the diffraction patterns even in the absence of approximate molecular replacement phases. Useful derivatives of macromolecules are normally obtained by soaking native crystals in solutions of a heavy-atom compound or by crystallization of the macromolecule from a solution containing the heavy atom. With this approach, the number of heavy atoms that bind to the macromolecule is largely a matter of chance, but the odds of obtaining a useful derivative for a typical macromolecule with these equilibrium techniques are sufficiently high that more sophisticated techniques are rarely needed. However, since ribosomal particles have a much larger surface area than any macromolecule solved by these techniques so far, there is a proportionately larger number of potential binding sites for isomorphous substituents. For this reason, it would clearly be preferable not to leave the binding of the heavy-atom clusters to chance.

An alternative to the equilibrium binding of isomorphous substituents to the crystalline molecule is the formation of a covalent derivative of the molecule at a specific site before crystallization. This approach often requires sophisticated synthetic techniques and time-consuming purification procedures, but it offers us a much better chance of obtaining interpretable changes in the diffraction pattern of a ribosomal particle. Moreover, specific derivatization of selected ribosomal components will ultimately be of considerable value in localizing the components and related functional sites in the three-dimensional structure of the ribosome.

Monofunctional reagents with a maleimido moiety as the reactive group have been prepared from both of the heavy-atom clusters described earlier. Since the clusters are rather bulky, the accessibility of the reactive group was varied by attaching it to the clusters with aliphatic chains of different lengths (Weinstein et al., 1989). The modified clusters were bound to specific sites on the ribosome before crystallization in one of two ways: (i) by direct reaction with chemically active groups on the surface of the ribosome or (ii) by covalent attachment to an isolated ribosomal component that was subsequently reconstituted into the ribosome.

For the first approach, conditions under which a small number of sulfhydryl groups are exposed on the surface of the ribosome were determined. We were able to find conditions under which only one to three sites were exposed on each of the ribosomal particles that could crystallize. For the 50S subunit from *H. marismortui*, for example, we could take advantage of previous studies which showed that the stability, compactness, and biological activity of halophilic ribosomes depend strongly on the concentrations of salts as well as on the delicate equilibrium between the monovalent and divalent ions in the medium (Shevack et al., 1985; H.-S. Gewitz, I. Makowski, S. Weinstein, U. Evers, A. Yonath, and H. G. Wittmann, unpublished data). To obtain an efficient reaction with both clusters, it was necessary to reduce the concentration of the KCl from 3 M, the concentration normally used to stabilize these ribosomes, to 1.2 to 1.5 M. To avoid disintegration of the subunits and depletion of ribosomal proteins at the KCl concentration required for the reaction, the concentration of Mg^{2+} was increased to 20 to 50 mM. Under these conditions, the halophilic ribosomes maintain their integrity for long periods of time and the clusters are bound predominantly to one site.

To limit the reaction of the maleimido group to the sulfhydryl groups of the ribosomal proteins, the binding was conducted at around pH 5.5. The 50S subunits of *H. marismortui* are crystallized and can be safely maintained at this pH. To improve the efficiency of the reaction, it was necessary to attach another aliphatic chain to the −SH groups on the surface of the particle, in addition to the reactive arm of the clusters. It was found that a spacer with a minimum length of about 10 Å between an −SH group on the ribosomal particle and the N atom of the maleimido group of the cluster was needed to bind between 0.5 to 1 equivalent of the gold cluster directly to the 50S subunits of *B. stearothermophilus*. All of the 50S subunits so obtained yielded crystals isomorphous with the native crystals.

Genetic and chemical procedures for obtaining ribosomes of *B. stearothermophilus* in which protein BL11 is missing were developed. The genetic proce-

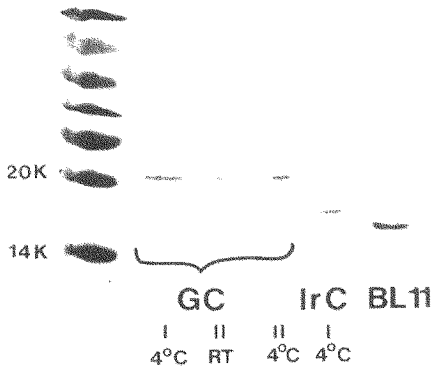

Figure 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of BL11-gold cluster (GC), BL11-iridium cluster (IrC), and BL11. I and II indicate derivatization of BL11 with short and long handles, respectively. The binding reactions were carried out at 4°C or room temperature (RT).

dures included the growth of the bacteria at 60°C in a medium containing thiostrepton (J. Schnier, H.-S. Gewitz, and T. Leighton, unpublished data). The mutated 70S ribosomes and their 50S subunits lacking protein BL11 formed crystals isomorphous to those of the wild-type particles (Yonath et al., 1986b; Müssig et al., 1989). These results demonstrate that protein BL11 is not involved in crystal contacts and that its absence does not induce gross conformational changes to either the 70S or the 50S particle. Similar attempts to crystallize 50S particles lacking protein BL12 failed, possibly because the absence of this protein influences the overall structure of the ribosome (Carazo et al., 1988).

Our initial efforts at the second approach to producing specifically modified ribosomal particles, namely, the derivatization of isolated ribosomal components, have involved protein L11 from *B. stearothermophilus* (BL11). BL11 is an example of a ribosomal component that undergoes reversible conformational changes upon isolation from the particle. In isolation, its −SH group is reactive only under denaturating conditions, whereas this group is exposed on the surface of the ribosome. Protein BL11 has been used to covalently attach heavy-atom clusters to specific sites on ribosomal particles from *B. stearothermophilus*. Both the gold and the iridium clusters could be quantitatively bound to isolated BL11 (Fig. 4). Binding of the clusters changes the mobility of the protein in sodium dodecyl sulfate-gels, and the corresponding bands were found in positions expected for a protein whose molecular weight is the sum of those of BL11 and of the clusters (Fig. 4). The modified protein was subsequently incorporated into mutant 70S ribosomes or 50S subunits lacking L11 to form specific, quantitatively modified particles. These modified particles are biologically active, even though a large heavy-atom cluster is attached to them. To place the size of the clusters into perspective, we note that the molecular weight of the undecagold cluster is 6,200, which is more than a third of the molecular weight of BL11 (15,500).

Both two-dimensional and three-dimensional crystals could be obtained from 50S subunits reconstituted with covalently labeled BL11 by using the same conditions under which the native particles crystallize (Müssig et al., 1989). Diffraction data of a quality comparable to that from the native crystals were collected from crystals of the modified particles, which were found to be isomorphous with the native crystals. These experiments demonstrate that it is possible to label ribosomes by specific covalent binding of heavy-atom clusters without introducing major changes in their crystallizability, integrity, conformation, or biological activity.

Removal of specific proteins from the ribosome can also be achieved by stepwise addition of compounds such as salts or alcohols to their storage buffers. We have used such compounds under mild conditions to make large-scale preparations of the ribosomal proteins as well as ribosomal core particles depleted of small numbers of proteins. In all cases, the detached proteins could be reincorporated into the depleted particles by partial reconstitution. Both we and others were able to obtain crystals of good quality from one of the isolated proteins, L6 from *B. stearothermophilus* or *T. thermophilus*. This result demonstrates that the isolation procedure was mild enough not to denature this protein (Gewitz et al., 1987; Sedelnikova et al., 1987).

In addition to the components of the native ribosome, any compound that forms a tight, specific complex with ribosomal particles can in principle be used as a carrier for the binding of heavy-atom clusters. Appropriate carriers may be antibiotics (Nierhaus et al., 1989) or DNA oligomers complementary to exposed single-stranded rRNA regions (Hill et al., 1986). Since most of the interactions of such carriers are well characterized biochemically, the crystallographic location of the heavy-atom clusters attached to such compounds will again provide information useful not only for phase determination but also for the localization of important structural and functional sites on the ribosome.

We have begun studies directed at crystallization of complexes between ribosomal particles and other components of protein biosynthesis whose locations are of particular interest. Small crystals of 50S sub-

units from *B. stearothermophilus* that retain a short nascent polypeptide and a tRNA molecule have been obtained recently (Table 1; Gewitz et al., 1988; Müssig et al., 1989). Although these crystals are not yet suitable for crystallographic analysis, our previous experiences with variations in crystallization conditions among different preparations of ribosomal particles make us optimistic that it will eventually be possible to produce useful derivatives of this complex by incorporating heavy-atom clusters into the oligopeptide. Another obvious target for the indirect attachment of heavy-atom clusters to ribosomal particles is tRNA. To facilitate crystallization of complexes of tRNA and ribosomal particles, we have determined conditions under which one molecule of acylated tRNA binds to 30S subunits and conditions under which two molecules of charged tRNA bind to 70S ribosomes from *T. thermophilus* (C. Glotz, H. J. Rheinberger, A. Yonath, and H. G. Wittmann, unpublished data).

A POSSIBLE ASSIGNMENT OF FUNCTIONAL SITES IN RECONSTRUCTED MODELS OF RIBOSOMAL PARTICLES

We have already described how we hope to use three-dimensional image reconstruction of two-dimensional crystals of ribosomal particles to assist in solving the phases of our three-dimensional crystals. However, because the reconstruction from two-dimensional arrays has intrinsic advantages over the more traditional electron microscopic study of single particles, the reconstructions are interesting in their own right as low-resolution images of ribosomal particles. Imaging studies of biological samples with the electron microscope share several limitations, regardless of whether one studies single particles or organized arrays. These limitations arise primarily from the difficulties of preserving biological specimens, in the high vacuum in the sample chamber of the microscope, from radiation damage and from complications to the interpretation of the images due to staining. The use of ordered arrays of particles differs fundamentally from the study of single particles in that image reconstruction is based on diffraction from large numbers of particles held in the same orientation by lattice contacts, whereas single-particle techniques involve either manual scoring or computer-aided averaging of limited numbers of selected particles. The selection of single particles for analysis is at best based on statistical correlations but has more often involved subjective judgment. Moreover, it is commonly observed in micrographs of samples of single particles that the particles tend to lie on the grid in a small number of preferred orientations; it remains an unsolved question whether contact with the grid distorts the projected images of the particles. The use of two-dimensional crystals has the consequence that any distortions which may be introduced by the electron microscope grid will be reduced or even eliminated by the interparticle crystal forces.

The growth of two-dimensional arrays of 50S subunits has been described elsewhere (Lake, 1979; Oakes et al., 1986; Yonath et al., 1986c). These arrays were grown within weeks or even months and yielded diffraction information that was only marginally suitable for reconstruction studies. Thus, the resulting reconstructions had limited resolution, and the models derived from them had very little detail (Clark et al., 1982; Oakes et al., 1986). More recently, we have been able to grow well-ordered sheets of ribosomal particles within a few seconds by using salts as crystallizing agents (Arad et al., 1987a; Piefke et al., 1986).

We were able to derive low-resolution models at 30 and 47 Å resolution, respectively, from tilt series of well-ordered arrays of both 50S subunits and 70S ribosomes from *B. stearothermophilus*, negatively stained with gold thioglucose (Yonath et al., 1987; Piefke et al., 1986; Arad et al., 1987b). The reconstructed models have volumes which agree well with those measured by other physical methods or calculated from the composition of *E. coli* ribosomes (Wittmann, 1983; Hardesty and Kramer, 1986). Even at low resolution, the reconstructed models display several striking features that had not been previously detected in procaryotic ribosomes. On the basis of the available biochemical information, we have suggested functional roles for some of the features detected in these reconstructions. We should emphasize, however, that a detailed assignment of functional sites on the ribosome will require structural information of much higher resolution.

Nearly 20% of the volume within the envelope of the 70S ribosome in our reconstruction has a density level comparable to that found for the stained regions between particles (Fig. 5). This empty space is large enough to contain two to three tRNA molecules with room to spare for other factors of protein biosynthesis, and thus it is an obvious candidate for the location of the various binding sites and enzymatic activities associated with the process of protein synthesis. The small and large ribosomal subunits are well separated in all reconstructions of whole ribosomes (Milligan and Unwin, 1986; Arad et al., 1987b; Wagenknecht et al., 1989), but the empty space between the subunits, which is clearly resolved in our studies (Fig. 5; see also Fig. 7), is less well

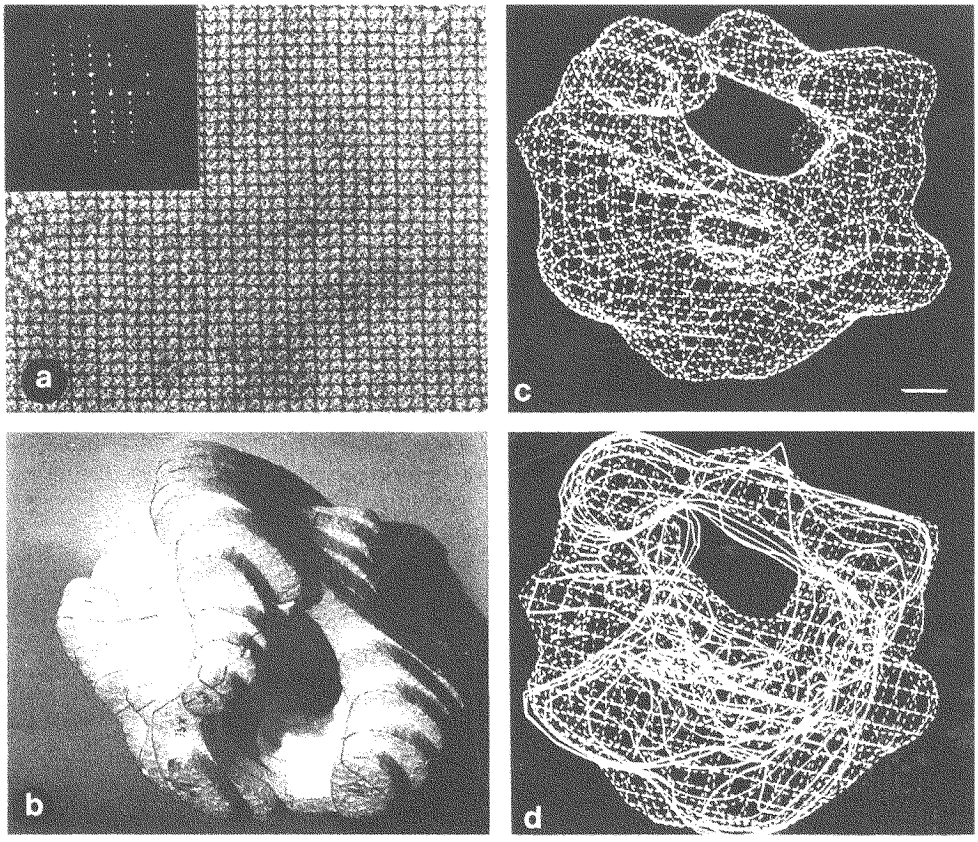

Figure 5. (a) Negatively stained (with gold thioglucose) two-dimensional sheet of 70S ribosomes from *B. stearothermophilus* and its diffraction pattern. (b) Physical model of one of the reconstructed images. (c) Computer graphic representation of another reconstructed model. (d) The images shown in panels b and c superimposed on each other. Both reconstructions were obtained at 47 Å. Bar, 20 Å.

defined in the reconstructions from two-dimensional sheets of 80S ribosomes (Milligan and Unwin, 1986) and from averaged images of single 70S ribosomes (Wagenknecht et al., 1989). It is likely that the poor definition of the empty space in the 80S ribosomes results from the lower resolution at which this reconstruction was performed and, in the single-particle reconstruction of the *E. coli* ribosomes, from shrinkage or collapse of the particles on the grid (Wagenknecht et al., 1989).

Another prominent feature of our reconstructions of negatively stained two-dimensional sheets of 50S subunits from *B. stearothermophilus* is a tunnel of about 100 Å in length and up to 25 Å in diameter through the 50S subunit (Fig. 6). It could also be detected in filtered images of the same sheets viewed unstained in the electron microscope at cryotemperature (Fig. 6; M. Giersig, unpublished data). Our observation of a tunnel in the 50S subunit from *B. stearothermophilus* is consistent with the results of image reconstruction of two-dimensional sheets of 80S eucaryotic ribosomes, which revealed a channel in the large subunit (Milligan and Unwin, 1986). More recently, a similar feature was found in the 50S subunit of *E. coli* ribosomes by Wagenknecht et al. (1989), who derived their model from averaged images of single 70S ribosomes that were selected by statistical criteria. The tunnel is clearly visible in all reconstructions of 50S particles (Yonath et al., 1987b) but is somewhat less well resolved in those of assembled ribosomes (Milligan and Unwin, 1986; Arad et al., 1987b; Wagenknecht et al., 1989), possibly because their tunnels are partially filled with nascent protein chains or because of the lower resolution.

We have tentatively located the large subunit of the 70S ribosome from *B. stearothermophilus* by manually fitting our reconstruction of the large subunit into the reconstruction of the complete ribosome, using interactive computer graphics (Fig. 7). The fitting was based both on similarities in the overall shapes of the models and on orientation of the tunnel. The overall agreement in the shapes of the 50S reconstruction and the part of the 70S reconstruction that was assigned to it is quite striking. However, there are two regions in which the two

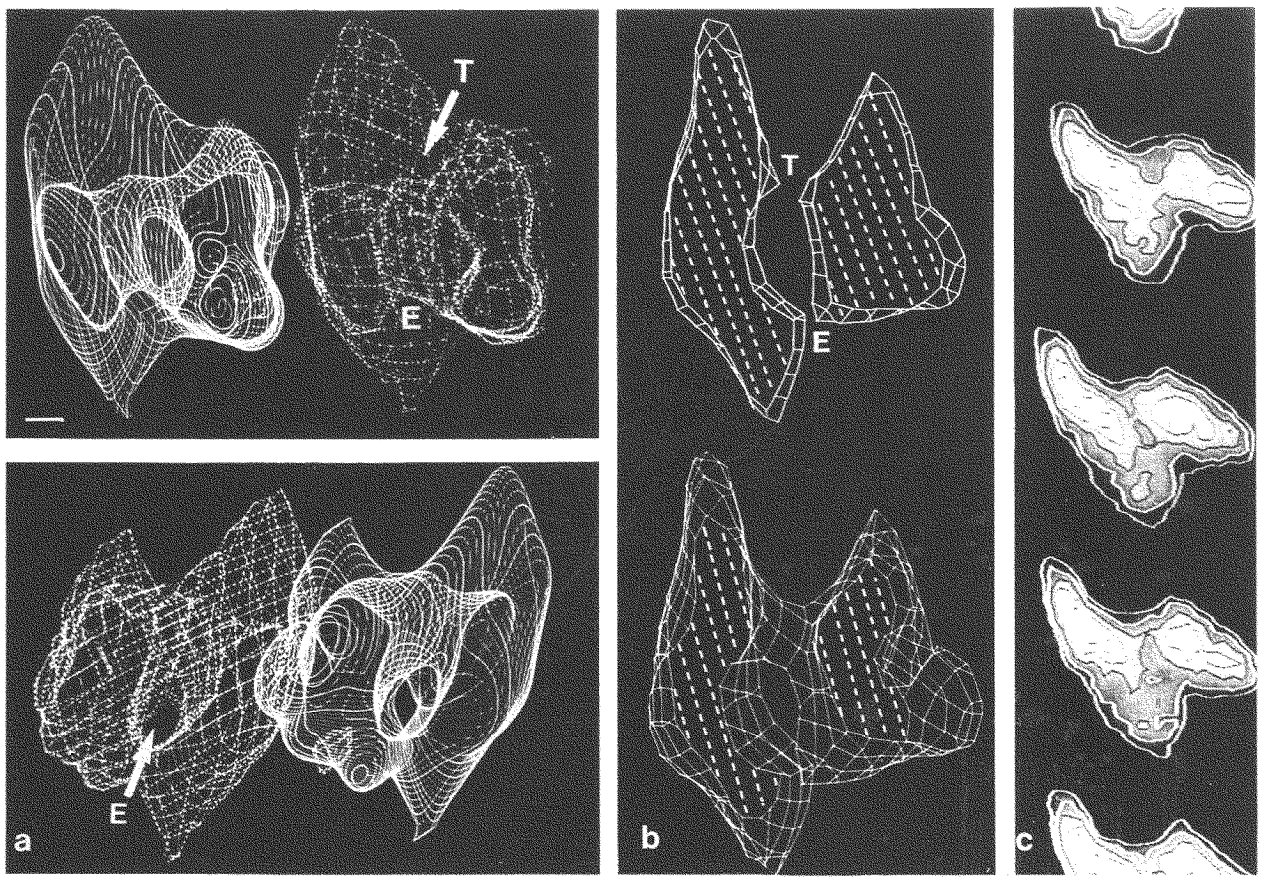

Figure 6. (a) Two computer graphic displays (as a net and in lines) of two views of the reconstructed model of the 50S subunit obtained from negatively stained (with gold thioglucose) two-dimensional sheets. (b) Two sections of thickness of 20 Å (top) and 40 Å (bottom) through the reconstructed model shown in panel a. (c) Filtered images of the unstained sheets of 50S subunits, grown as described by Arad et al. (1987a) and viewed at cryotemperature. Panels a and c were reconstructed or filtered at 28 Å. The entrance to (T) and exit from (E) the tunnel are indicated. Bar, 20 Å.

models differ slightly (Fig. 7). At this stage, it is not clear whether these differences reflect conformational changes occurring upon association of the subunits or whether they simply reflect the differences in the resolution of the two reconstructions.

Since two-dimensional sheets of 30S subunits sufficiently well ordered for diffraction studies have not yet been grown, a reconstruction of the small subunit free from the potential artifacts of single-particle reconstruction techniques is not available. However, after fitting the model of the 50S subunit into that of the 70S ribosomes of *B. stearothermophilus*, we could examine the interface between the two subunits as well as deduce the approximate shape of the small subunit within the 70S particle (Berkovitch-Yellin et al., in press [b]). The subunits are clearly separated by the empty space discussed above (Fig. 7). We will refer to this region as the intersubunit space.

The resulting model for the 30S particle is shown in Fig. 8. There is some similarity between the model of the small subunit so obtained and that observed by visualization of single particles (reviewed in Wittmann, 1983, and Hardesty and Kramer, 1986). However, isolated 30S particles appear somewhat wider in most electron micrographs and in reconstructions from single particles than in our model. Since we can compare our model of the 30S subunit only with models derived with single-particle techniques, artifacts such as flattening of the isolated particles on the grid might contribute to the differences observed. As was the case in our comparison of the reconstructed 50S and 70S particles, these differences may also result from conformational changes in the subunits when they associate to form the 70S ribosome.

More than a decade ago (Malkin and Rich, 1967; Blobel and Sabatini, 1970; Smith et al., 1978), as well as more recently (Rayabova et al., 1988; Yen et al., 1988; Evers and Gewitz, unpublished data), it was observed that the ribosome protects a growing polypeptide chain from enzymatic digestion until the

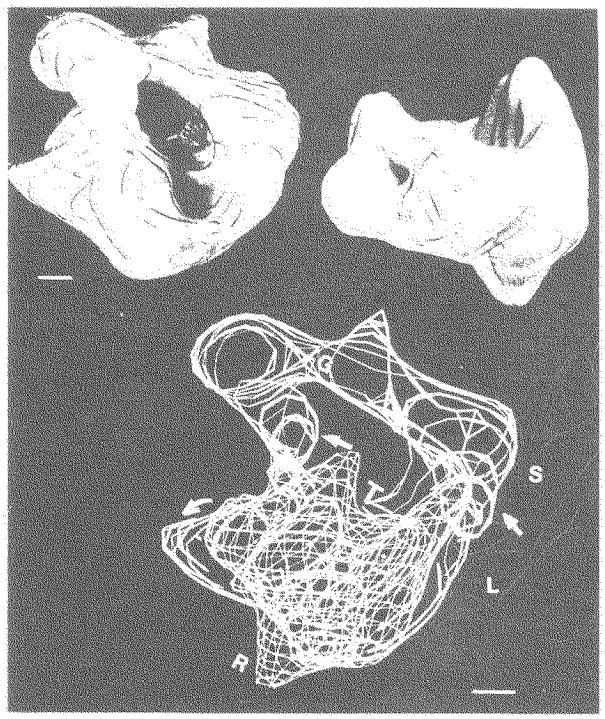

Figure 7. (Top) Physical models of the reconstructed images of the 50S subunit (right) and the 70S ribosome (left). (Bottom) Superpositions of computer graphic displays of the outlines of the reconstructed models of the 70S ribosome (in lines) and of the 50S ribosomal subunit (drawn as a net). L and S, 50S and 30S subunits, respectively; G, a groove, rich in RNA, in the small subunit; T, cleft and entrance to the tunnel in the 50S subunits. The extra density (R) may be a consequence of the different resolutions at which the two models were reconstructed (30- and 47-Å resolution, respectively). The arrows indicate a possible direction for a cooperative movement of the regions where differences in density between the model of the isolated 50S subunit and that in the 70S ribosome were found. Bar, 20 Å.

nascent protein can fold sufficiently to develop its own resistance to degradation. The tunnel in the 50S subunit leads from the intersubunit space in the 70S ribosome, which we presume to be the site of the actual protein biosynthesis process, to a location compatible with the exit site of the nascent chain identified by immunoelectron microscopy (Bernabeu and Lake, 1982). The tunnel is long enough to accommodate a polypeptide of a length comparable to that found by biochemical methods to be protected by the ribosome (25 to 40 amino acids) in any conformation, and its diameter (up to 25 Å) is sufficient to pose no obvious restrictions to the sequence of such peptides. It can also provide enough room to amino acids to which bulky groups such as biotin are bound (Kurzchalia et al., 1988). The tunnel thus provides a plausible exit pathway and a natural explanation for the observed protection of the nascent polypeptide chain.

To test our hypothesis that the tunnel serves as the exit pathway for a growing polypeptide chain, we have begun a series of experiments aimed at investigating the properties of the tunnel. By identifying compounds that can be bound to the tunnel, we should be able to map the chemical environment within the tunnel as well as identify additional potential carriers for heavy-atom clusters that could provide new tools for phase determination. We have found that despite the excess positive charge of the ribosomal proteins, a newly formed chain of polylysine can be tightly bound to the large subunits of *B. stearothermophilus* (Gewitz et al., 1988), suggesting that the walls of the tunnel may be formed in part by rRNA. Newly synthesized chains of polyphenylalanine are known to bind to 50S subunits from *E. coli* (Gilbert, 1963), *B. stearothermophilus*, and *H. marismortui* (Gewitz et al., 1988), suggesting also that some regions of the tunnel walls may be hydrophobic. These experiments have already led to the crystallization of the complexes between 50S subunits and oligopeptidyl-tRNA described earlier.

The ability of the ribosome to protect a growing protein is sequence independent. Besides naturally occurring proteins (Malkin and Rich, 1967; Yen et al., 1988) and the polylysyl and polyphenylalanyl chains mentioned above, sequences rich in proline, obtained by coding with poly(C) or random polymers of poly(C-A) or poly(C-U), are also found to be protected by the ribosome (Evers and Gewitz, unpublished data). The various polypeptides differ in their affinities for the ribosome. Interestingly, polyproline, which is synthesized at a much lower rate than the

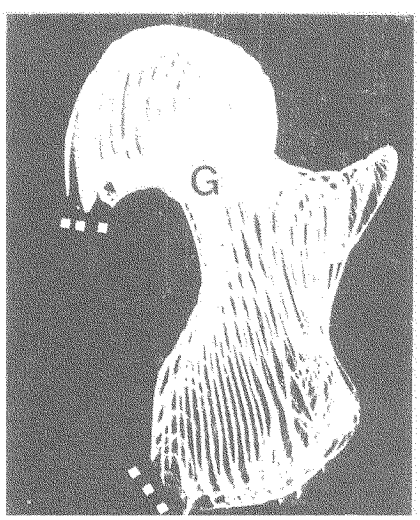

Figure 8. Outline of the 30S ribosomal subunit obtained by subtracting the 50S subunit from the 70S subunit. The regions of interaction with the 50S subunit are indicated by (▪▪▪). G, Groove in the small subunit. Bar, 20 Å.

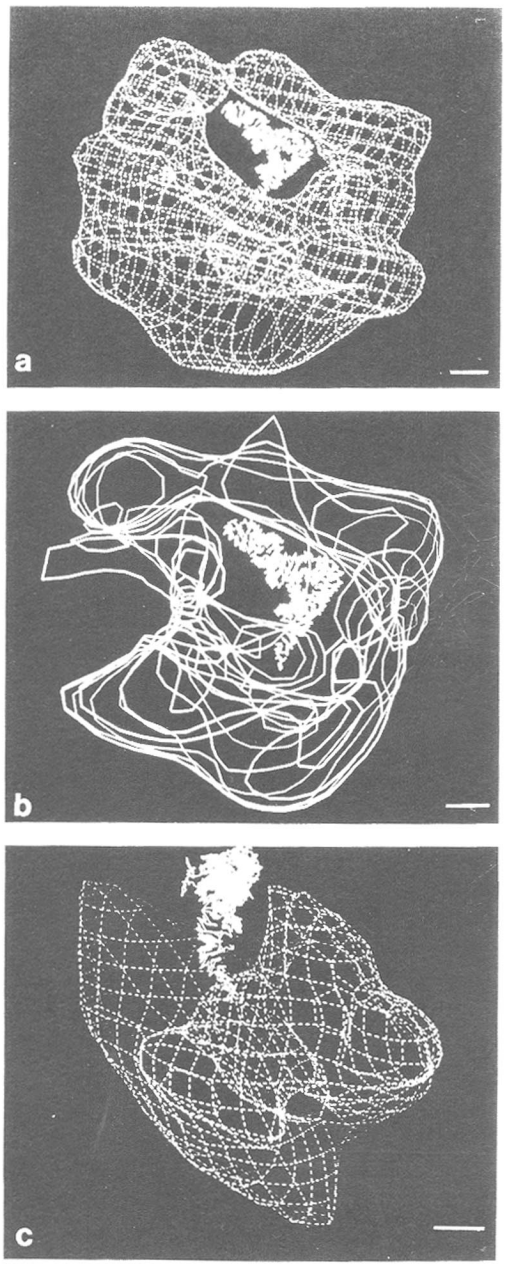

Figure 9. (a and b) Speculative model showing how a tRNA molecule may be integrated into the two reconstructed models of the 70S ribosome shown in Fig. 5. The anticodon loop is in the proximity of the groove (G in Fig. 7 and 8) on the 30S subunit and the CCA end points at the entrance to the exit tunnel. (c) Possible arrangement between the tRNA molecule and the 50S subunit. The envelope of the whole 70S ribosome shown in panels a and b has been removed for clarity. Bar, 20 A.

other polypeptides, dissociates slowly from the ribosome, possibly because it has the most rigid conformation.

It was recently shown by immunoelectron microscopy that the N terminus of a nascent chain can be detected in two distinct patches on the surface of the ribosome (Rayabova et al., 1988). One patch is close to the subunit interface, and the second is at the other end of the particle, suggesting that the N terminus might be exposed at a different site when only 2 to 6 residues have been synthesized than when the chain is 40 or more residues long. It is conceivable that during the initial stage of protein biosynthesis, the first few amino acids of the newly formed protein do not enter the tunnel. Only in cases when the growing chain finds its way into the tunnel would the process of protein biosynthesis continue. This hypothesis may explain why only 40 to 50% of a given population of ribosomes are active in protein biosynthesis, whereas almost all of them bind tRNA (H. J. Rheinberger and K. H. Nierhaus, personal communication).

Uranyl acetate, which is commonly used as a stain for electron microscopy of biological specimens, acts as a partially positive stain and is incorporated into the ribosomal particles chemically. rRNA, the ribosomal component most likely to interact with uranyl acetate, can be detected by comparing reconstructed images obtained from two-dimensional arrays that have been stained by purely negative stain (e.g., gold thioglucose) and by uranyl acetate. RNA-rich regions were detected at the interface of the small and the large subunits, in agreement with studies performed on unstained arrays of eucaryotic ribosomes (Milligan and Unwin, 1986). Another RNA-rich region was found on the 30S subunit (Arad et al., 1987b; Yonath and Wittman, 1989b) in the vicinity of a groove that is clearly visible in the model derived from our reconstructions of 50S and 70S particles. In accordance with biochemical and model-building experiments (Brimacombe et al., 1988), we have tentatively identified this groove as the mRNA-binding site. The intersubunit space in the 70S ribosome can easily accommodate a tRNA molecule with its anticodon close to the mRNA binding site and its CCA terminus positioned such that the peptidyl group may extend into the tunnel. A tRNA molecule placed in this orientation would probably be able to interact with the walls of the intersubunit space directly; such interactions might account for noncognate interactions between the tRNA and the ribosome. A speculative model-building experiment in which a tRNA molecule has been manually positioned in the intersubunit space is shown in Fig. 9. The figure demonstrates that the intersubunit space is large enough to accommodate one or two more tRNA molecules, with some room left for other compounds, e.g., elongation factors.

CONCLUSIONS

From the early stages of this work, it has been clear that the conventional techniques of macromolecular crystallography would not be adequate for determining the structures of ribosomal particles. We have devised an approach that combines phase determination methods of macromolecular crystallography with cryotemperature techniques to obtain higher-resolution data and the exploitation of the extensive information available on the genetic and chemical properties of ribosomes for the rational design of isomorphous derivatives of ribosomal particles by using heavy-atom clusters. These tools have already enabled us to overcome a number of the difficulties associated with crystallographic analysis of ribosomal particles that were once thought to be insurmountable. This broad-based approach, together with recent advances in the instrumentation for X-ray crystallography, leaves no major conceptual obstacle to determination of the three-dimensional structure of the ribosome, although a considerable amount of work remains to be done before this goal is realized. In anticipation of the success of these efforts, we have already begun crystallization studies directed at obtaining crystals in which the ribosome is trapped in different functional states (Bennett and Huber, 1984). In this way, one can use a number of static "snapshots" of a dynamic system such as the ribosome to visualize the sequence of events of the dynamic process and to aid in the interpretation of studies by other techniques.

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