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Crystallography of ribosomes: Attempts at decorating the ribosomal surface

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Abstract

Crystals of various ribosomal particles, diffracting best to 2.9 Å resolution were grown. Crystallographic data were collected from shock frozen crystals with intense synchrotron radiation at cryo temperature. For obtaining phase information, monofunctional reagents were prepared from an undecagold and a tetrairidium cluster, by attaching to them chemically reactive handles, specific for sulfhydryl moieties. Heavy-atom derivatives were prepared by a specific and quantitative binding of the undecagold cluster to an exposed sulfhydryl prior to the crystallization. To create potential binding sites on the halophilic and thermophilic ribosomal particles, which yield our best and most interesting crystals, exposed reactive moieties were inserted, using genetic and chemical procedures. In order to choose the appropriate locations for these insertions, the surfaces of the ribosomal particles were mapped by direct chemical determination of exposed amino and sulfhydryl groups.

Keywords: Haloarcula marismortui; Thermus thermophilus; Ribosomes; X-ray crystallography; Methylation; Sulfhydryls

1. Introduction: crystallographic studies

1.1. Why crystallography?

Ribosomes are the universal cell organelles facilitating the enzymatic translation of genetic information into proteins. Bacterial ribosomes contain three RNA chains (of a total of over 4500 nucleotides), which account for two thirds of their mass, and 58–73 different proteins, depending on the source. Bacterial ribosomes are called 70S, according to

Abbreviations: r-Proteins stands for ribosomal proteins and rRNA for ribosomal RNA. E, B, T, and H in front of a sedimentation co-efficient: 70S, 50S or 30S shows the bacterial source (*Escherichia coli; Bacillus stearothermophilus; Thermus thermophilus* and *Haloarcula marismortui*, respectively) of the whole ribosome and its large and small subunits. The same stands for the individual ribosomal proteins, the names of which are composed of a letter L or S (showing that the protein is of a large or a small subunit), and a running number, according to its position on two-dimensional gels of these subunits. The symbol: HmaL# indicates that the particular halophilic protein is homologous to protein L# from *E. coli*

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their sedimentation coefficient, and consist of two subunits of unequal size (30S and 50S) which associate upon the initiation of protein biosynthesis. The molecular weights of the three ribosomal particles, 70S, 50S and 30S are 2.3, 1.45 and 0.85 million daltons, respectively.

Although intensive biochemical, biophysical and genetic studies shed light on several functional and evolutionary aspects of protein biosynthesis and provided some information on the gross structural features and the in situ internal arrangement of a few ribosomal components (for review see [1,2]), it is clear that for the illumination of the molecular mechanism of protein biosynthesis a reliable molecular model is essential. To this end we initiated X-ray crystallographic studies.

1.2. The superiority of the halophilic and thermophilic ribosomes

It was commonly assumed that intra-cellular organelles which require flexibility to function, and therefore their populations may acquire a large spectrum of conformational states, cannot crystallize. Nevertheless, as a result of a systematic search for conditions under which homogenous populations of ribosomal particles are prepared and kept for periods long enough to allow crystallization, over 18 crystal forms of ribosomal particles were grown by us [3,4]. The list shown in Table 1 focuses on those crystal forms which are suitable for crystallographic studies at various resolution limits. As seen, all well diffracting crystal forms grown so far are of ribosomal particles from extreme thermophilic [5] and halophilic ribosomal bacteria [6], whereas the traditionally studied, hence well characterized *E. coli* ribosome yielded so far only micro-crystals [7]. We relate the success in crystal growth of the halophilic and thermophilic ribosomes to the higher stability and lower sensitivity and flexibility of the thermophilic and halophilic ribosomes, properties which were most likely imposed by the harsh environmental conditions. In fact, it was found that the quality of the crystals is directly linked to the level of extremeness in the natural environment of the bacterium.

Many aspects of crystallography of biological macromolecules are associated with sophisticated biochemistry. Several examples are given below: (i) for many steps in structure determination, especially those involving modifications, homogeneous preparations of pure particles are required, as these are the key for the production of quality crystals; (ii) for exploring the dynamics and the functional activity, well defined populations of carefully designed complexes mimicking specific stages in the biosynthetic pathway are a prerequisite; (iii) the common experimental procedures for phase determination depend on the production of specific derivatives.

Table 1
Three-dimensional crystals of ribosomal particles

Source	Grown form	Cell dimensions (Å)	Resolution (Å)
70S t.t.	MPD [a]	$524 \times 524 \times 306$; $P4_12_12$	App. 20
70S t.t. + mRNA and tRNA [b]	MPD	$524 \times 524 \times 306$; $P4_12_12$	12
30S t.t.	MPD	$407 \times 407 \times 170$; $P42_12$	7.3
50S H.m.	PEG [a]	$210 \times 300 \times 581$; $C222_1$	2.9
50S t.t.	AS [a]	$495 \times 495 \times 196$; $P4_12_12$	8.7
50S B.st. [c]	A [a]	$360 \times 680 \times 920$; $P2_12_12$	App. 18
50S B.st. [c,d]	PEG	$308 \times 562 \times 395$; 114°; C2	11

[a] MPD, PEG, AS, A = crystals were grown by vapor diffusion in hanging drops from solutions containing methyl-pentane-diol, polyethyleneglycol, ammonium sulfate or low-molecular-weight alcohols, respectively.

[b] A complex including 70S ribosomes, 2 molecules $phetRNA^{phe}$ and an oligomer of 35 uridines (as mRNA).

[c] Same form and parameters for crystals of large ribosomal subunits of a mutant (missing protein BL11) of the same source and for modified particles with an undecagold cluster.

[d] Same form and parameters for small crystals of a complex of 50S subunits, one tRNA molecule and a segment (18–20 mers) of a nascent polypeptide chain, diffracting currently to 10–12 Å.

B.st. = *Bacillus stearothermophilus*, t.t. = *Thermus thermophilus*, H.m. = *Haloarcula marismortui*.

30S = small ribosomal subunits, 50S = large ribosomal subunits, 70S = whole ribosomes.

As even the most carefully prepared populations of the favorable research object, the *E. coli* ribosome, are known to be heterogenous, the absolute requirement for quantitization in crystallographic studies led to its substitution by the more robust particles from thermophilic or halophilic bacteria [8,9]. Thus, the proven superiority of halophilic and thermophilic ribosomes for structural studies stimulated conceptual revisions as to the suitability of the inherently sensitive and unstable *E. coli* ribosome not only for crystallography but also for biochemical studies.

1.3. Crystallographic studies

So far, all crystals of ribosomal particles were obtained from biologically active particles. The chemically modified or biochemically depleted particles which could be crystallized belong also to this category. It was found that for obtaining crystals from the treated particles, the starting preparations must be not only homogeneous but also highly active. Furthermore, it is noteworthy that the integrity and the activity of the crystalline ribosomal particle are maintained for extremely long periods, despite the natural instability of the ribosomes and their tendency to disintegrate even under favorable conditions. Aiming at elucidating the structure of the ribosomal particles at a conformation closest to that required for their activity, special procedures were designed for data collection from crystals immersed in solutions of compositions mimicking those of the physiological environment within the cells [10,11].

The weak diffracting power of the ribosomal particles and the large unit cell dimensions dictate the performance of virtually all the crystallographic studies with synchrotron radiation of a high brightness. At ambient temperature the diffraction of the ribosomal crystals decays within the first instances of irradiation. Assuming that this decay is caused by propagating free radicals which are being formed by the radiation, we developed special procedures for eliminating their movement within the irradiated crystals, by lowering the temperatures to cryogenic limits. Consequently, data are collected at about 85–100 K from shock frozen crystals.

Special protocols were designed for the precooling and freezing steps which accommodate the problematic features of the ribosomal crystals: fragility, sensitivity and extremely thin edges [10,11]. It was found that properly shock frozen crystals yield data of higher resolution than those measured at ambient temperatures, as they can be measured at longer exposures. They also exhibit the same mosaic spread as the average values obtained at ambient temperatures, and diffract with no observable decay for periods allowing the collection of more than a full data-set from an individual crystal. Furthermore, the irradiated crystals can be stored for months and still maintain their diffracting power.

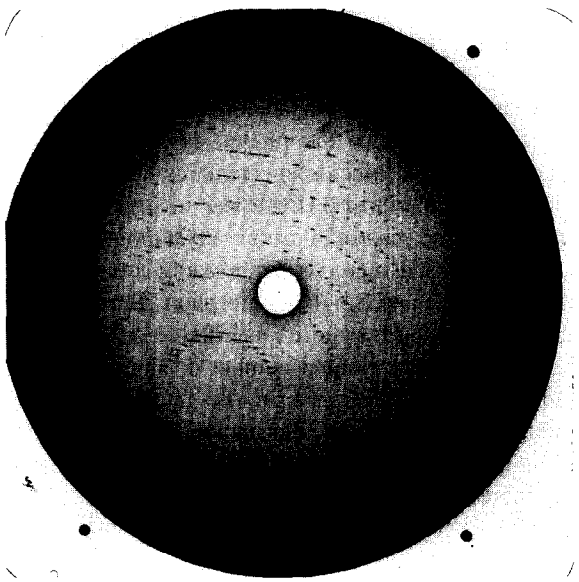

Fig. 1. A rotation photograph of a crystal of H50S, obtained at 90 K at station F1/CHESS, operating at 5.3 GeV and 50 mA. Crystal -to-film distance = 220 mm, collimator = 0.1 mm; wave length = 0.9091 Å.

The crystals of the large ribosomal subunits of *H. marismortui* (H50S) diffract to the highest resolution obtained so far from crystals of ribosomal particles: 2.9 Å (Fig. 1). They are of reasonable mosaicity (0.2–0.3°) and adequate mechanical stability [6]. They reach an average size of $0.3 \times 0.3 \times 0.05$ mm, have cell dimensions of $210 \times 300 \times 570$ Å and $C\bar{2}22_1$ symmetry. Their crystallographic data are of quality comparable to that obtained from crystalline proteins of average size. Thus, for above 55% completeness, the typical values for R-merge(I) at 6 Å are in the range of 5–10% [11] and at 3 Å, R-merge(I) = 12–15%.

1.4. Phasing by specific labeling on the ribosomal surface

Phases are crucial for the construction of electron density maps. Since the phases cannot be directly measured, their elucidation remains the less predictable and the most difficult task in structure determination. In macromolecular crystallography the commonly used methods for phasing are MIR and SIR (Multiple and Single Isomorphous Replacement, respectively), both require quantitative attachment of heavy atoms at a limited number of sites within the unit cell. The difference in the intensities of the diffraction patterns of the native and derivatized crystals are being exploited for phase determination, therefore the added moieties have to be chosen according to their potential to induce measurable signals.

For proteins of average size (e.g. with molecular weights of 15–80 KDa), useful isomorphous derivatives consist of one or two heavy-metal atoms. Due to the large size of the ribosome, to reach the required electron density an ideal compound to be used for the derivatization should consist of a compact cluster of a proportionally larger number of heavy atoms, linked directly to each other. Two such compounds, an undecagold cluster, with a total molecular weight of 6200 daltons and a tetrairidium cluster, of a molecular weight of 2300 daltons (Fig. 2) have been developed [3,12,13,26]. Although never used before for this purpose, we have designed monofunctional reagents of the two clusters, to facilitate specific derivatization of the ribosomal particles or of compounds with a high affinity to the ribosomes, by co-valent binding of these clusters.

Assuming reasonable isomorphism and a full oc-

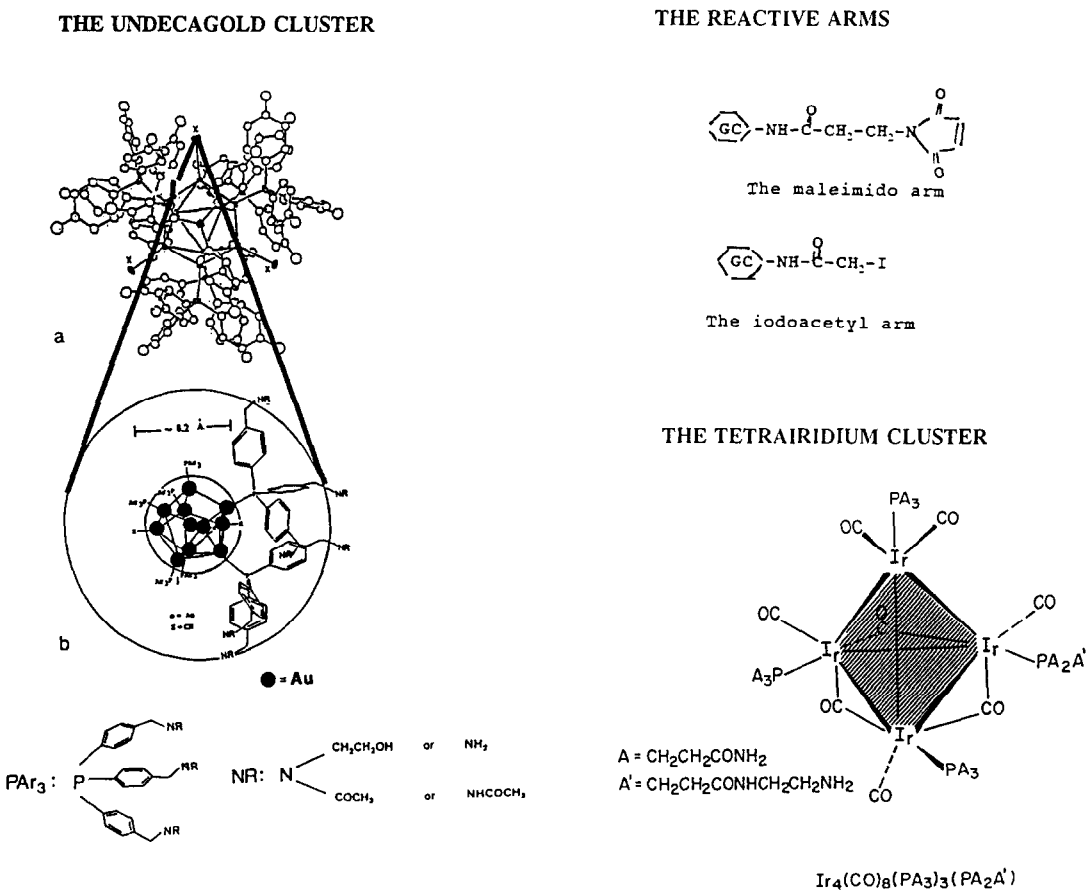

Fig. 2. Schematic representations of the undecagold and the tetrairidium clusters [12,13,26].

cupancy of the gold cluster in one site on each of the 30S, 50S and 70S particles, the average change in diffraction intensities is expected to be about 19%, 15% and 11%, respectively. Hence, provided these requirements are fulfilled, the undecagold cluster is likely to induce measurable differences between the intensities of the reflections of the native crystals and those of the derivatized ones and lead to phase information. In fact, simulation studies [15] confirmed the above calculations and indicated adequate phasing power (N. Volkmann and H. Bartels, to be published). As the core of the undecagold cluster is about 8.2 Å in diameter [3,12,13], 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 [3,26], and can thus be treated as a single heavy atom to somewhat higher resolution ranges.

Derivatives of crystals of biological macromolecules are routinely obtained by soaking crystals in solutions of the heavy-atom compounds or by co-crystallization of the macromolecule together with the heavy atom. Using these procedures, the number of heavy atoms that bind to the macromolecule is largely a matter of chance, but the chances 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 an extremely large and complex surface area, it would clearly be preferable not to leave the binding of the heavy atom to pure chance. An alternative procedure for derivatization is co-valent binding of the heavy-atom compound to the molecule at one or a few specific sites before crystallization. This approach requires sophisticated synthetic techniques and time-consuming purification procedures, but it offers a much better chance of obtaining a unique derivative. Specific derivatization of selected ribosomal components may have a considerable value not only in phasing the crystallographic data, but also in localization of specific sites on the ribosomes, information which may be indispensable for the understanding of the function of the ribosome.

To facilitate quantitative and site specific derivatization, a monofunctional reagent was developed from the undecagold cluster (Fig. 2). The first targets for binding the cluster to the ribosomes were exposed free sulfhydryls [4,12,13]. To enable the binding of the clusters, we increase their accessibility by attaching to them a chemically reactive arm of 4.5–12 Å in length with either a maleimido or iodoacetyl moiety at its end. The latter was designed to avoid the chirality introduced by the reaction with the double bond of the maleimido moiety.

Preliminary derivatization experiments were performed with the undecagold cluster with its longer arm on 50S subunits from *Bacillus stearothermophilus*. One ribosomal protein, BL11, was removed by mutagenesis [14] or by a stepwise addition of salts [15]. It was found that the cores lacking protein BL11 crystallize isomorphously with the native ones, indicating that the removal of this protein caused neither major conformation changes in the ribosome, nor disturbances in the crystal's network.

The undecagold cluster was bound quantitatively to the isolated ribosomal protein, exploiting its only cysteine. The modified protein could be reconstituted into cores of mutated ribosomes, lacking it, obtained by growing the cells on the antibiotics thiostrepton [14]. The crystals of the so obtained fully derivatized particles yielded data of reasonable quality, and the low resolution electron density map phased by this undecagold cluster is of reasonable quality and contains features of the size and shape similar to those seen for the large ribosomal subunits by electron microscopy (H. Bartels, to be published).

2. Biochemical efforts: surface mapping and decorations

2.1. Is a straight-forward extension of eubacterial biochemistry to halophilic systems possible?

The suitability of the undecagold cluster for phasing data collected from crystals of the 50S subunits from *B. stearothermophilus*, encouraged extension of this procedure to the ribosomal particles from *Haloarcula marismortui* and *Thermus thermophilus*, which yield crystals of a much higher quality. However, this task was hampered by severe difficulties. Because of the significant resistance of the halophilic ribosomes to mutations [16] no protein-depleted core particles could be produced by growing the bacteria on antibiotics. Also, the chemical methods used for quantitative splitting of the *E. coli* ribosomal proteins were found unsuitable for the halophilic and

thermophilic systems. Furthermore, in contrast to the ease of the incorporation of the gold-cluster bound protein BL11 into depleted cores of *B. stearothermophilus*, protein HmaL11, the presumed halophilic homolog of BL11, can be reconstituted into core particles only when its sulfhydryl group is free.

Hence, it became clear that the potential heavy-atom binding sites on the surfaces of the halophilic and thermophilic ribosomes should be inserted by chemical or genetic procedures. For choosing appropriate locations for these insertions one can benefit from the advanced stage of the sequencing of the halophilic r-proteins [17] and from studies on surface mapping, performed either by limited proteolysis [18] or by direct chemical determination of exposed amino and sulfhydryl groups (see below). As the genes of some of the halophilic and thermophilic proteins have been cloned [19,20], the insertion of chemically reactive side chains into the surface of the ribosome became feasible.

Four ribosomal proteins were removed quantitatively by dioxane from the large halophilic subunits (H50S) and two from the small ones (H30S), all of which could be fully reconstituted into the depleted core particles. As mentioned above, one of these split proteins, HmaL11, binds reagents specific to –SH groups, but the derivatized protein could not be incorporated into the core particles. In this way cores of 50S subunits, depleted of one protein (HmaL11), were obtained. The relative ease of the detachment of HmaL11 from the halophilic as well as the homologous protein from various bacterial ribosomes poses the interesting question as to the role of this protein in the translational process. Clearly, it displays rather weak in situ interactions. As for the case of BL11, the ribosomal subunits lacking protein HmaL11 crystallize under the same conditions as native H50S and show apparent isomorphism with them, suggesting that the depletion of this protein does not cause major conformation changes, and that protein HmaL11 is not involved in the crystal's network. Therefore it is a most suitable candidate for the insertion of exposed cysteines.

2.2. *Some thoughts about the nature of the ribosomal surface*

An exact definition of the ribosomal surface of the halophilic and thermophilic ribosomes is still not possible, as some parameters influencing the ribosomal compactness have not been identified yet. In fact, even the surface of the *E. coli* ribosome, which is considered to be the best characterized one, is still not totally known, mainly because of its inherent conformational heterogeneity. Thus, it has been recently suggested that almost all properties assigned to the *E. coli* ribosome were actually identified only for subsets of ribosomal populations, and may not reflect the overall behavior (for a review see [9]).

Although it was suggested by Sir F. Crick almost three decades ago that the original ribosome was made entirely of RNA, until recently it was assumed that the catalytic activities of the ribosome are carried out solely by the ribosomal proteins and the ribosomal RNA has more passive roles, such as providing the scaffold of the ribosomal network. In addition to the general tendency to assign enzymatic activities to proteins rather than to RNA molecules, there were sound reasons for these assumptions. Thus, electron microscopy as well as contrast methods, which by their inherent nature are not suitable for detecting fine structural details, showed that most of the rRNA is located in the core of the ribosomal particles. Researchers extrapolated that the internal core of the ribosome consists mainly of rRNA, and that this core is covered by the ribosomal proteins.

The recent evidence from affinity labeling experiments accompanied by the demonstrations of the catalytic abilities of RNA in several biological systems, the accumulation of data showing substantial conservation in some rRNA regions and the blocking of several enzymatic activities by hybridization with anti-sense DNA or by antibiotics which are known to hydrolyze RNA, indicate that a significant amount of rRNA is exposed, and stimulated the design of experiments challenging the above dogma. Thus, the ribosomal functions are no longer attributed solely to the ribosomal proteins [21,22], and several distinct ribosomal functions have been attributed to the rRNA. A striking example is the GTPase center, which is associated with a highly conserved stretch of 23S RNA which binds protein L11. Another example is the peptidyl transferase activity, for which the suitability of the thermophilic bacteria was clearly demonstrated. Solid results were obtained only when thermophilic ribosomes were used, whereas the *E. coli* ribosomes gave rise to ambiguous results, mainly

because of the poor quantitization. Thus, only 20–40% of the original activity of a model-assay for peptidyl transferase was retained in *E. coli* 50S subunits, from which most of the protein mass was removed, whereas the *T. acuarticus* ribosomes exhibited 80% of their original activity after depletion of about 95% of the ribosomal proteins [21].

2.3. DNA oligomers, complementary to exposed rRNA

Probing exposed single-stranded rRNA with complementary DNA oligomers gained considerable popularity recently in mapping the ribosome surface. In studying the initiation step, it was found that AUG and dATG containing oligomers promoted the non-enzymatic binding of fmet–tRNA with similar properties and formed initiation complexes which are fully reactive with puromycin [23]. In parallel, for illuminating the formation of the initiation complex, the competition between a variety of cDNA oligomers and poly(U), poly(AUG), tRNAphe, tRNAfmet and the initiation factors were monitored [24]. In addition, anti-sense DNA was proven to be instrumental in monitoring dispositions of normal and altered *E. coli* 16S rRNA [25].

Exposed single-stranded rRNA segments which may be complemented by DNA oligomers have been located on halophilic and thermophilic ribosomes. The targeted regions include bases 1125–1158 of 23S RNA, homologous to those from *B. stearothermophilus*, which in the wild type are masked by protein BL11 but become exposed in the mutant lacking this protein; the last 14 nucleotides from the 3′ end of the 16S RNA (the vicinity of the 'Shine Dalgarno' position) of *T. thermophilus*; bases 2646–2667, the 'alfa-sarcin binding site' on 23S RNA of *T. thermophilus* and *H. marismortui* and bases 1422–1432, the 'thiostrepton binding site', of the latter [4].

2.4. Probing exposed ribosomal proteins by mild methylation and acylation

The surface ribosomal proteins are more manageable than the exposed rRNA. Therefore they are more suitable for the crystallographic aims of introduction of specific and quantitative modifications which should lead to phasing and consequent structure determination.

Earlier accessibility studies concentrated on limited proteolysis. These experiments were optimized first for *E. coli* or other eubacterial ribosomes [18]. Attempts to extend them to the halophilic ribosomes were only partially successful and sometimes misleading. Thus, the halophilic ribosomes require high salinity for maintaining their compactness and their active configuration, whereas the conditions suitable for the enzymatic reaction of all the commercially available enzymes are milder. Therefore, even when using the most robust proteolytic mixtures, partial unfolding of the halophilic ribosomes could occur. Such unfolding may not effect dramatically the overall sedimentation coefficient, showing that the gross integrity of the ribosomal particles is being kept, but at the same time, opening-up of the compact active conformation and consequently exposing internal residues cannot be ruled out. Therefore, the aim of the surface mapping experiments, which are designed to provide fine and accurate details, cannot be achieved.

An illuminating example is given below. The significant contribution of the Mg^{2+} ion to the activity of ribosomal particles from all sources has been discovered long ago. Eubacterial ribosomes are strictly dependant on a minimum of a few mM of Mg^{2+} for their function. The case of the halophilic ribosomes is more intricate, as a delicate balance between the mono- and di-valent ions is required. Following the dependence of the number of exposed sulfhydryls of H50S on the relative concentrations of the potassium and the magnesium ions showed that up to 15 sulfhydryls become exposed upon lowering the KCl concentration from 3 to 1.5 M in the presence of less than 10 mM Mg^{2+}. However, even at low KCl (1.2–1.5 M), the presence of 20–25 mM Mg^{2+} was sufficient to keep the number of exposed sulfhydryls close to two, the minimal value [4]. The increase of exposed sulfhydryls upon lowering the Mg^{2+} concentration indicates partial opening (or unfolding) of the structure of the ribosome, and shows that a few mM of Mg^{2+} have the same influence on the compactness of the halophilic ribosomes as 1–1.5 M KCl do. It is noteworthy that labeling the cysteines of H50S which are exposed under the conditions which maintain the compact-

ness, did not hamper the functional activity of these ribosomal particles as well as their crystallization [3,12,13].

We assumed that if carefully designed, chemical procedures have the potential to be safer and may provide more concrete information than enzymatic ones. We first chose methylation and acylation, and this manuscript is focused on these experiments. We aimed at probing accessible primary amino groups for two reasons. The first is the preparation of binding sites for heavy atoms, especially the monofunctional reagent of a compact and soluble tetrairidum cluster (Fig. 2 and refs. [12,13,26]). The second aim was the prospect of improving the quality of our crystals, according to the procedures published recently for a muscle protein [27] and lysozyme [28]. There it was shown that although methylation of the exposed lysines of these proteins introduced only minor changes in their structures, these subtle modifications were found to be crucial for obtaining high quality crystals.

As mentioned above, the core of the tetrairidium cluster has a smaller diameter (5 vs. 8.2 Å) and is somewhat more symmetrical than the undecagold cluster (Fig. 2). Therefore it can be treated as a 'single scatterer' at around 3.5–5 Å. Since the electron density of the iridium cluster is not as high as that of the undecagold cluster, for obtaining measurable signals, multiple, rather than a single derivatization is recommended. As the iridium has a significant anomalous effect, crystallographic data will be collected from the modified particles at various wave-lengths, especially near the iridium absorption edges. This should facilitate the determination of the phases either by direct methods (disregarding the ribosome, and treating the clusters as a 'small molecule') or, for cases with a small number of binding sites, by traditional MIR, SIR or MAD (Multiple Isomorphous Dispersion).

Lysines may be the right candidates for binding the tetrairidium cluster molecules at their expected resolution range (3.5–5 Å). Two kinds of reagents were designed for probing the ribosomal exposed lysines. For the binding of propionyl groups we are using N-hydroxysuccinimide ester of propionic acid. The methylation, which means binding of two methyl groups per lysine residue, is performed by the reagents formaldehyde and borane-dimethylamine adduct. As in all species the number of ribosomal lysines is by far larger than that of the cysteines, the design of the experiments for the identification of the exposed lysines is bound to be more sophisticated and the analysis of the results should be more critical. As expected, only in a few occasions the methylation reached completeness, and generally only partial information could so far be obtained, but it is clear that the extent of the methylation was found to be dependent on the nature of the ribosomal particles.

In typical experiments 25–60% of the lysines of the halophilic large ribosomal subunit, H50S, those that yield our best diffracting crystals, became methylated. The extent of methylation is dependant on the experimental conditions. Following the published procedures [27,28] resulted in the highest level of methylation (55–60%) and in the growth of microcrystals from the methylated particles. By reducing the concentration of the formaldehyde (up to 40% of the reported one), a lower extent of methylation was obtained. The H50S particles with 25% methylation yielded crystals, which on several occasions reached an unusual large size, but in other cases only microcrystals were obtained. These results indicate that: (a) at least two-thirds of the lysines of the halophilic large ribosomal subunits are exposed; (b) the fact that no crystals grew above a specific limit of methylation suggests that some of the exposed lysines participate in the crystal network, therefore their partial or full methylation reduces the quality or inhibits the growth of crystals; and (c) that the products of the reaction are not homogenous, however, a fair amount of the resulting population is of properties with a potential to yield improved crystals.

The small halophilic ribosomal subunit, H30S, behaves differently. Upon methylation under the original conditions [27,28] as well as under milder ones, substantial disintegration was observed. In fact, with the exception of protein HS6 and maybe HS13, all other ribosomal proteins were either partially or fully detached. A partial list of the fully detached proteins includes HS2, HS3, HS7, HS15, HS19, HS21 (numbering according to ref. [29]). Thus, it seems that the halophilic small ribosomal subunits are less stable than the large ones, similar to the lower stability and higher sensitivity observed for several small eubacterial ribosomal subunits [3,30].

T30S was readily methylated to a slightly higher extent (about 70%) than that found for the H50S subunit. Further derivatization (by acylation) of the methylated particles was no longer possible, thus showing the complete blocking of exposed amino groups. Therefore, the easily obtained close-to-total methylation of the isolated proteins of this subunit indicate that about 30% of the lysines are engaged in internal contacts, presumably with the ribosomal RNA.

So far no crystals could be grown from the modified T30S particles, and only 22% of the original biological activity of the particles was maintained. The failure to produce crystals from the modified subunits may be linked either to gross conformational changes which effect the crystallizability and the activity or from alterations in the regions engaged in the crystalline contacts which may involve the surface lysines. It is noteworthy that the higher stability of the large ribosomal subunits was demonstrated also for the thermophilic ribosomes. Thus, 67% of the original activity was maintained after methylation, although one protein, TL7 seemed to be detached from the T50S. It is noteworthy that a higher degree of acylation was found for the small thermophilic subunit than for the large one. Although, as seen below, the acylation reaction is less defined than the methylation, this finding accords well with the earlier finding indicating the general higher flexibility of the small ribosomal subunits [3,30].

Conditions for specific or quantitative acylation could not yet be determined, although most of the ribosomal proteins became acylated to various extents. Further attempts to shed light on the acylation were carried out using H50S subunits. Intact particles were first acylated and then some of their proteins were split using dioxane [4]. The acylated proteins were reconstituted into non-acylated cores lacking them. A significant level of reconstitution was observed, but we still could not determine the exact stoichiometry of the acylation and it is not clear whether the reconstitution was quantitative.

Fig. 3. shows typical two-dimensional gels of the ribosomal proteins of intact and acylated T30S subunits. These gels were obtained according to the scheme described in ref. [31], when an acidic pH difference (between 4 to 5) was kept on the first dimension, whereas a constant value of pH 4 was maintained in the second. The differences in the migration properties and spot profile of the intact and modified ribosomal proteins stem from the nature of the acylation reaction. The lysines of the natural ribosomes are protonated at acidic pH whereas because of the acylation, the free amino groups of the lysins are blocked and the proteins loose their ability to become charged by protonation.

Inspection of these gels show that the basic proteins, e.g. TS19 and TS20, undergo substantial acylation and appear as very broad spots, making the estimation of the extent of methylation almost impossible. However, parallel radioactivity experiments

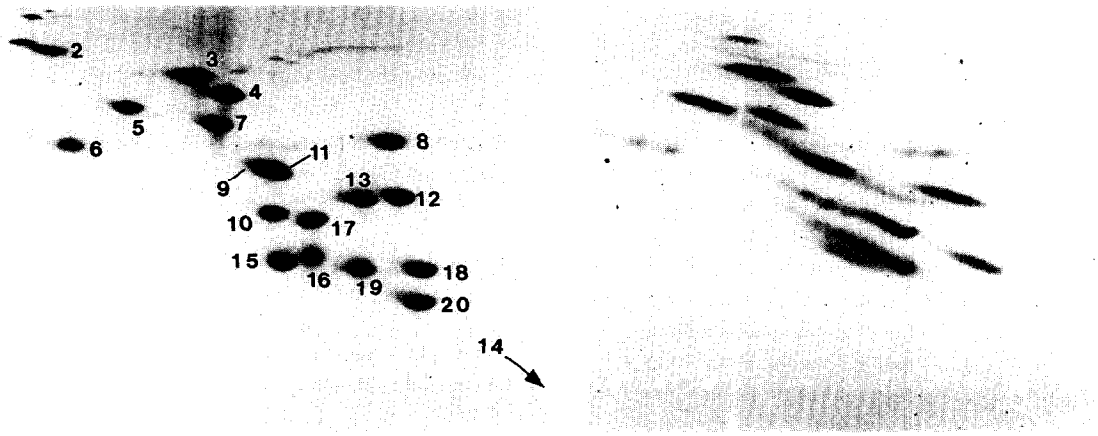

Fig. 3. Two-dimensional PAGE of the ribosomal proteins of T30S. Left: intact subunits, the numbering is according to ref. [20]. Right: after treatment with N-Hydroxysuccinimide propionic acid ester, at an excess of 150 equivalents.

indicated high extent of acylation throughout the ribosomal proteins of this subunit. Unfortunately, a satisfactory non-radioactive method for the determination of the extent of the acylation of T30S is still not available. Noticeable is the problem of the separation between the spots on the 2D gels. Thus, proteins TS14, TS15 and TS13 appear as a continuous ladder, and spots TS9 and TS11 overlap, as found also for T30S particles in which the cysteines have been modified. Noteworthy is the disappearance of the spot corresponding to protein TS2. As no solid evidence for detachment has been found, the disappearance of the stop of this protein from the vicinity of its original location may be explained by extensive modification which varied substantially its migration properties, so that it could not be detected on the gels.

3. Concluding remarks

As seen, the complexity, sensitivity to external conditions and the inherent conformational flexibility of all ribosomal particles, including those believed to be rather robust ones (those purified from the bacterium living in the Dead Sea), hampers most of the experiments aimed at extracting accurate information about the nature of the surface of the ribosomes. However, some valuable information has already been obtained. Thus, both the acylation experiments led to general results, although the products of these reactions have not been fully characterized.

In principle, these experiments could be exploited as a diagnostic tool for the exact localization of surface lysines. The accuracy of such diagnostics depends mainly on the knowledge of the sequences of the ribosomal proteins and of the level of commitment. Thus, for obtaining accurate mapping information, studies should be pursued thoroughly to their limits, at which every individual labeled lysine is being identified. For this aim the labeled proteins have to be isolated to purity, sequenced and compared with native proteins. Reaching this target for all the modified lysines is bound to be a very lengthy and tedious task, and we doubt if the prospective benefit for our crystallographic studies justifies it. On the contrary, for our aims valuable information can be obtained at a significantly lower detail. Thus, the mere differentiation between the exposed and buried lysines and the distinction between the readily modified and less reactive ones are of a significant experimental value. In addition, the second target for methylation, the improvement of the quality of the crystals should not be forgotten, and the occasional growth of very large crystals from the methylated H50S particles stimulates more effort in this direction.

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