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Derivatization of ribosomes and of tRNA with an undecagold cluster: crystallographic and functional studies

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An undecagold cluster was covalently attached to whole ribosomes and to their small and large subunits prior to their crystallization. X-ray crystallographic data were collected from crystals of the first two. The same cluster was bound to $tRNA^{phe}$ from *E. coli* at base 47. It was found that the modified tRNA molecule binds to the ribosome and can be aminoacylated by its cognate synthetase. The gold cluster modified $tRNA^{phe}$ may be used for phasing diffraction data of crystals of complexes containing it, mimicking defined states in the process of protein biosynthesis.

1. Introduction

Of all organelles in the living cell, only the ribosome has thus far been crystallized. Ribosomes are supramolecular assemblies responsible for one of the most fundamental life processes: translation of the genetic code into proteins. A typical bacterial ribosome (called 70S ribosome, according to its sedimentation coefficient) has a molecular weight of 2.3×10^6 Da. It is composed of some 57 different proteins and three chains of rRNA (with a total of about 5500 nucleotides, accounting for two thirds of its weight), distributed in two independent subunits of unequal size (called 50S and 30S subunits, with molecular weights of 1.45×10^6 and 0.85×10^6 Da, respectively) which associate upon initiation of protein synthesis.

X-ray crystallography is the most suitable method for elucidating the three-dimensional structure of biomacromolecules. It requires crystals of a high quality and adequate size. Ribosomes are notoriously unstable and flexible, hence difficult to crystallize. However, a systematic search for suitable sources for ribosomes and for appropriate crystallization conditions led to the formulation of conditions under which crystals of several ribosomal particles, diffracting up to 3 Å [1], were grown (table 1, fig. 1 and in refs. [1–3]). At cryo temperature these crystals can be irradiated by a synchrotron X-ray beam for periods long enough for the collection of a complete data set from a single crystal without apparent radiation decay [4].

A common method for phasing diffraction data from crystals of biological macromolecules is the multiple isomorphous replacement (MIR), which requires the preparation of at least two derivatives, usually by introducing one or a few electron-dense atoms to the crystalline lattice at distinct locations. These added atoms should be dense enough to cause measurable changes in the diffraction pattern while keeping the crystal structure isomorphous to that of the native molecule. For proteins of average size a useful isomorphous derivative consists of one or two heavy-metal atoms. Due to the large size of the

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Table 1
Characterized three-dimensional crystals of ribosomal particles

Source	Growth from [a]	Cell dimensions (Å)	Resolution [b] (Å)
70S *Thermus thermophilus*	MPD	$524\times524\times306$; $P4_12_12$	≈ 20
70S *Thermus thermophilus* + mRNA&t-RNA [c]	MPD	$524\times524\times306$; $P4_12_12$	≈ 15
30S *Thermus thermophilus*	MPD	$407\times407\times170$; $P42_12$	7.3
50S *Halobacterium marismortui* [e]	PEG	$210\times300\times581$; $C222_1$	3.0
50S *Thermus thermophilus*	AS	$495\times495\times196$; $P4_12_12$	8.7
50S *Bacillus stearothermophilus* [d]	A	$360\times680\times921$; $P2_12_12$	≈ 18
50S *Bacillus stearothermophilus* [d),c]	PEG	$308\times562\times395$; 114°; C2	≈ 11

[a] MPD, PEG, A, AS: crystals were grown by vapor diffusion in hanging drops from solutions containing methyl-pentane-diol (MPD), polyethyleneglycol (PEG), ammonium sulphate (AS) or low molecular weight alcohols (A).
[b] "Resolution" refers to the highest resolution for which sharp diffraction spots could be consistently observed; in many instances we could not collect useful crystallographic data to this resolution.
[c] A complex including 70S ribosomes, 1.5–2 equivalents of $PhetRNA^{phe}$ and an oligomer of 35 ± 5 uridines, serving as mRNA.
[d] 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.
[e] Same form and parameters for crystals of a complex of 50S subunits, one tRNA molecule and a segment (18-mers to 20-mers) of a nascent polypeptide chain.

ribosome, an ideal compound for its derivatization should consist of a proportionally larger number of heavy atoms, linked directly to each other in the most compact fashion.

Derivatives of crystals of biological macromolecules are routinely obtained by soaking the crystals in solutions of the heavy-atom compounds. Employing this equilibrium technique, the production of useful derivatives is a matter of chance, which may be sufficiently high for regular proteins, but not for ribosomal particles due to their extremely large and complex surface area. Therefore alternative procedures for derivatization were developed, based on quantitative covalent binding of a heavy atom compound at a specific site prior to crystallization. Although this approach requires complex and time-consuming synthetic procedures, it should lead to unique-site derivatives which may have a considerable value not only in phasing but also in the localization of the cluster-bound ribosomal components, information which may be indispensable at later stages in the course of structure determination.

We are using a cluster containing a core of eleven gold atoms (with a diameter of 8.2 Å), surrounded by hydrophilic organic groups, chemically linked to form a well-defined compound with an overall diameter of 22 Å [5]. This undecagold cluster (GC) combines high electron density with water solubility, and can be prepared as a monofunctional reagent specific to sulfhydryl moieties [5–7]. Since the gold cluster is rather bulky, its accessibility was enhanced by attaching to it a maleimido group through aliphatic chains of varying lengths. In some cases the –SH groups on the surface of the ribosomal particles were also extended in a similar manner. It was found that a spacer of 10–20 Å between the –SH group and the cluster allows better binding [6,7].

The monofunctional gold cluster was attached either to the surface of the intact ribosome or to an isolated ribosomal protein which was subsequently incorporated into core particles, missing this protein. An example is the derivatization of the 505 subunits from *Bacillus stearothermophilus*. The gold cluster was bound to the sulfhydryl group of the isolated ribosomal protein BL11, and the modified protein was reconstituted with core particles of a mutant, lacking only this protein. Although the molecular weight of the

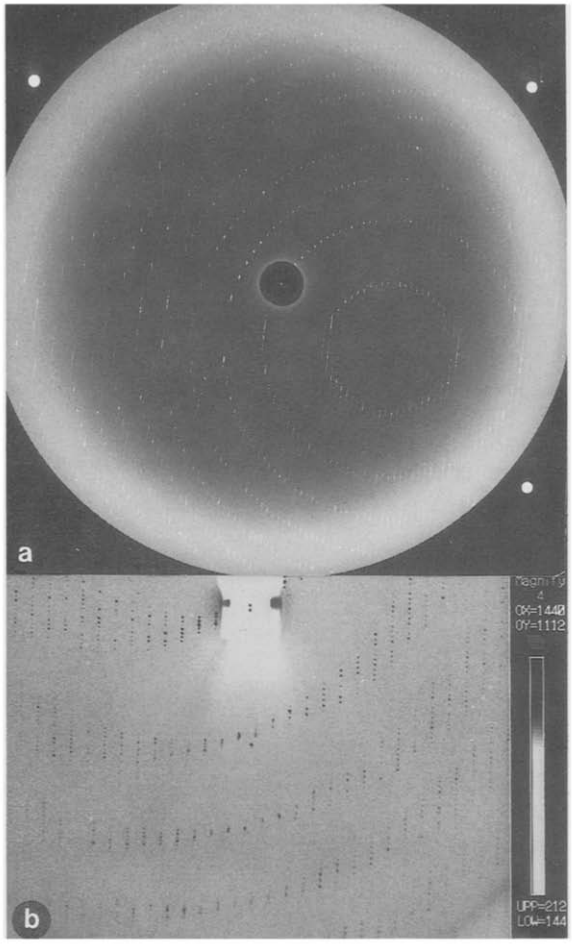

Fig. 1. X-ray 1.5° (a) rotation and (b) Weisenberg diffraction patterns of crystals of 50S subunits of *Haloarcula marismortui (halobacterium marismortui)*, grown as described in ref. [1], soaked in a solution with a composition similar to the natural environment within the bacteria (3M KCl, 0.5M ammonium chloride and 0.05M magnesium chloride). Prior to data collection, the crystals were gradually immersed in a cryogenic solution, containing besides the above mentioned components 18% ethyleneglycol. After about 30 min, the crystals were flash-cooled to around 90 K, and kept at this temperature throughout data collection. The patterns were recorded with synchrotron X-ray beam at (a) station F1/CHESS on film (exposure time = 10 min; crystal to film distance = 280 mm; wavelength = 0.92 Å) and at (b) station BL6/KEK/PF, on a double-sensitive image plate (curtesy of Dr. N. Sakabe), with a wavelength of 0.9019 Å, for 24 min exposure time. Crystal to film distance: 573 mm.

cluster is almost half of that of the protein (6200 versus 15500), and although the binding of the cluster was carried out under denaturation conditions (6M urea), upon incorporation into the mutated ribosomal core, the modified protein refolded to its regular in situ conformation. In this way we obtained specific and quantitatively modified particles, which could be crystallized [7].

In addition to the crystals of isolated ribosomal particles, crystals were obtained from complexes of ribosomes mimicking defined stages in the biosynthetic cycle, containing cofactors and products of the biosynthetic reaction. Elucidation of the structures of these complexes should shed light on the dynamic aspects of the molecular mechanism of protein biosynthesis. So far, two types of such complexes were crystallized: one containing 70S ribosomes, mRNA (as a chain of 35 uridines) and two *E. coli* $tRNA^{phe}$ molecules [8]; the second, composed of 50S subunits with a fragment of the nascent protein chain and one molecule of tRNA [2,9]. Besides the complexes described above, conditions were determined for stoichiometric binding of $tRNA^{phe}$ to several ribosomal particles [10].

Since $tRNA^{phe}$ is a part of all these complexes, it is an obvious target for indirect attachment of heavy-atom clusters to ribosomal particles. As most of the interactions of tRNA with the ribosome are well characterized biochemically, crystallographic determination of the locations of the heavy-atom clusters attached to it should assist phasing and, at the same time, provide information about the interactions of tRNA with the ribosome.

In this manuscript we report our crystallization attempts and crystallographic studies on derivatized ribosomal particles, describe the derivatization of $tRNA^{phe}$ and discuss its potential contribution to the phasing of X-ray diffraction data from complexes of ribosomal particles, mimicking defined stages in protein biosynthesis.

2. Experimental procedure

Ribosomal particles were obtained as described in ref. [11].

The gold cluster (GC) was prepared as described in ref. [5] . For the production of an arm,

specific for –SH groups, the monofunctional cluster was reacted with β-maleimidopropionic acid-N-hydroxysuccinimide ester, as described in ref. [7].

Quantitative analysis of the extent of binding of the CC was carried out by neutron activation [7] or by radioactivity, using ^{14}C (20 cpm/pmol) GC [12].

Free sulfhydryl groups, exposed on the surface of the ribosome, were detected by their reaction with radioactive markers, such as N-ethylmaleimide (NEM), parachloromercurobenzoate (PCMB) and iodoacetamide, followed by polyacrylamide gel electrophoresis of the ribosomal proteins, as described in ref.[7].

The monofunctional gold cluster with its extended arm was attached to free sulfhydryls on the surface of the ribosome as in ref. [7].

An exposed extended sulfhydryl was introduced to $tRNA^{phe}$ from *E. coli* as follows: 20 μl of 0.1M iminothiolane were reacted with 10 nmol $tRNA^{phe}$ (Boehringer), in 1M triethanol amine, 1M KCl, 0.01M $MgCl_2$, pH = 8.6, at 4°C, for 4 h. The monofunctional gold cluster, carrying a maleimido group, was attached to the newly introduced –SH as in ref. [7], at 4°C, pH = 6, for 2 h.

Separation of gold cluster bound $tRNA^{phe}$ ($tRNA^{phe}$–GC) from non-reacted molecules was performed by ethanol precipitation, followed by preparative HPLC on a reversed-phase column (for details, see fig. 2). A BARSPEC detector, with special features for the characterization of peaks by their spectrum, was used.

$tRNA^{phe}$–GC was visualized by dark field scanning transmission electron microscopy using the EMBL Cryo-STEM [13]. Specimens of 20 μg/ml were applied to thin carbon foil of approximately 30 Å thickness and freeze-dried. Images consisting of 1024^2 pixels of 2.34×2.34 Å^2 were recorded at a dose of approximately 200 electrons/Å^2 (acceleration voltage 100 kV).

$tRNA^{phe}$–GC was bound to 30S ribosomal subunits from *Thermus thermophilus* as follows: 30 pmol of 30S in a final volume of 100 μl of 0.05M tris-HCl (pH 7.5), 0.015M magnesium acetate and 0.16M ammonium chloride, were incubated for 20 min at 37°C, in the presence or in the absence of 0.375 mg/ml poly(U). ^{14}C (20 cpm/pmol) radioactive $tRNA^{phe}$–GC [12] was used for binding in a molar excess of 3–4. The extent of binding was checked after sucrose gradient centrifugation (10–35% sucrose in 30mM magnesium acetate and 75mM ammonium chloride and 10mM Tris buffer, pH 7.5) at 21 K for 18 h in SW40 rotor (4°C).

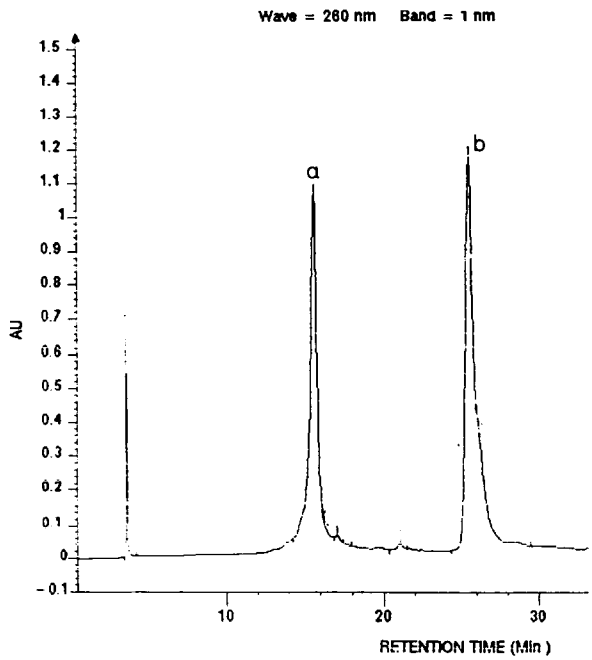

Fig. 2. HPLC of the reaction mixture of the binding of the gold cluster to $tRNA^{phe}$ Chromatographic conditions: reversed phase C4 column (Aquapore BU-300, 0.46×25 cm), 1 ml/min, combined linear and step gradient elution with Buffers A and B, from 0% to 100% Buffer B in 50 min. Buffer A: 0.4M NaCl, .20mM magnesium acetate, 20mM ammonium acetate, pH = 5.5. Buffer B: as A containing 60% methanol (v:v). (a) 15.58 min, $TRNA^{phe}$; (b) 25.81 min, $tRNA^{phe}$–GC.

$tRNA^{phe}$–GC was aminoacylated with phenylalanine as in ref. [14]

Models of tRNA–GC and its possible interactions with the ribosome were constructed on the E&S computer-graphics screen, using the crystallographically determined coordinates of a similar gold cluster [15] and of $tRNA^{phe}$ [16]. The package FRODO [17] was used for the display.

3. Results and discussion

3.1. Crystals of gold cluster bound ribosomal particles

3.1.1. Diffraction data from crystals of 50S subunits of B. stearothermophilus

As mentioned in the introduction, 50S subunits from *B. stearothermophilus*, reconstituted with gold-cluster labeled BL11, yielded two-dimensional sheets and three-dimensional crystals under the same conditions used for the crystallization of native particles [7]. X-ray diffraction data, of quality comparable to those obtained from the native crystals, were collected from crystals of the modified particles. Difference Patterson maps, computed from the diffraction data of the derivatized and the native crystals included features which could be interpreted as originating from the bound cluster [18]. 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 and that there are significant differences between the diffraction data of the native and of the modified ribosomal particles.

3.1.2. Derivatization of intact thermophilic and halophilic ribosomal particles

Conditions were defined for minimizing the number of exposed sulfhydryl groups which can bind the gold cluster, at different yields, to 1–3 sulfhydryls on the surface of each ribosomal particle which has been studied by us so far [2]. Almost quantitative binding of the cluster to a single site on the 70S ribosome and on the 30S subunit from *T. thermophilus* were achieved, and the modified particles yielded crystals which are smaller than, but isomorphous with the native ones. X-ray crystallographic data have been collected from both forms at cryo temperature [19].

The gold cluster was also bound, at a rather low yield (35–65%), to the surfaces of 50S subunits from *Haloarcula marismortui (Halobacteriummarismortui)* and *B. stearothermophilus*. Procedures for the enrichment of the number of modified particles in the resulting populations are being designed [20]. In parallel, attempts to bind the gold cluster to other chemically reactive groups on the ribosome are in progress [21].

3.2. Binding the undecagold cluster to tRNA^{phe}

We established the chemical conditions for binding the gold cluster to tRNA^{phe} from *E. coli* at base 47, which is a modified 3-(3-amino-3-carboxypropyl) uridine nucleoside (ACP3U). This base contains a relatively reactive primary amino group, which is exposed to the solvent. We used iminothiolane to convert the amine to a sulfhydryl moiety and to extend the functional group of the tRNA molecule in order to facilitate effective binding of the bulky gold cluster. The monofunctional maleimido gold cluster was reacted with this newly introduced –SH under mild conditions in order to maintain the native structure of the tRNA molecule.

Indications for the binding of the gold cluster to the tRNA^{phe} molecules were obtained by HPLC and by the characteristic Uv-visible spectrum of the gold cluster [5]. Quantitative analysis of the extent of binding of the gold cluster was carried out either by neutron activation [7] or by measuring the amount of the radioactivity of the ^{14}C gold cluster, prepared by us [12]. Up to 70% of the tRNA^{phe} were converted to a GC derivative. It was found that this yield could not be increased even at a high excess of both iminothiolane and GC. Since the tRNA^{phe} recovered from the reaction mixture by HPLC (fig. 2) and treated again as described above failed to bind the GC, we assume that commercial tRNA^{phe} includes molecules without the special base at position 47. The GC modified tRNA^{phe} was purified and separated from the non-reacted tRNA^{phe} molecules by ethanol precipitation, followed by preparative HPLC on a reversed-phase column (fig. 2). Analysis of the purified product by neutron activation and by using the radioactive ^{14}C GC [12] indicated an equimolar ratio of the GC to tRNA^{phe}. Gel electrophoresis showed one band, migrating slower than that of native tRNA^{phe} The UV–visible spectrum of the product was identical to that of an equimolar mixture of tRNA^{phe} and GC [5]. We found tRNA^{phe}–GC to be rather stable, less than 5% decomposition

was detected after incubation at 60°C at neutral pH for 1–2 h by HPLC.

$tRNA^{phe}$–GC was visualized by dark field scanning transmission electron microscopy using the EMBL Cryo-STEM [13]. Most of the molecular images acquired this way included a sharp bright spot (fig. 3) typical for the undecagold cluster [22].

Using radioactive $tRNA^{phe}$–GC [12], we found that the modified molecule could be aminoacylated with phenylalanine by the corresponding synthetase with the same rate and yield as the native molecule. In addition, we found that $tRNA^{phe}$–GC binds to 30S ribosomal subunits from *Thermus thermophilus* with the same stoichiometry as found for native $tRNA^{phe}$, in the presence and the absence of poly(U) (0.85 and 0.6 respectively).

These results show that a substantial modification in base 47 of $tRNA^{phe}$ can be tolerated by its synthetase. This modification also does not hamper the binding of the $tRNA^{phe}$ molecule to the 30S subunits. For testing possible modes for the interactions of $tRNA^{phe}$–GC with the ribosome, we assumed that during protein biosynthesis, the tRNA molecules bridge between the codon on the mRNA, which binds to the 30S subunit, and the tunnel in the 50S subunit which was suggested to be the path of the nascent protein [8]. We performed speculative model-building experi-

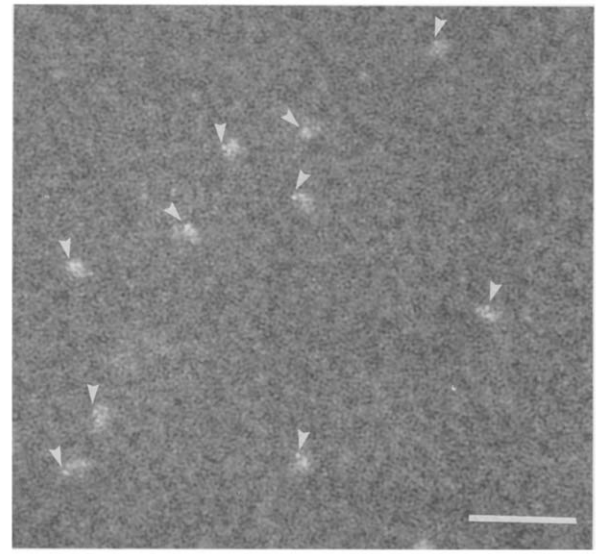

Fig. 3. $tRNA^{phe}$–GC in STEM dark field. Bar = 200 Å. The bright-sharp spots (marked by white arrows) indicate the cluster. The image was low-pass filtered in order to reduce noise.

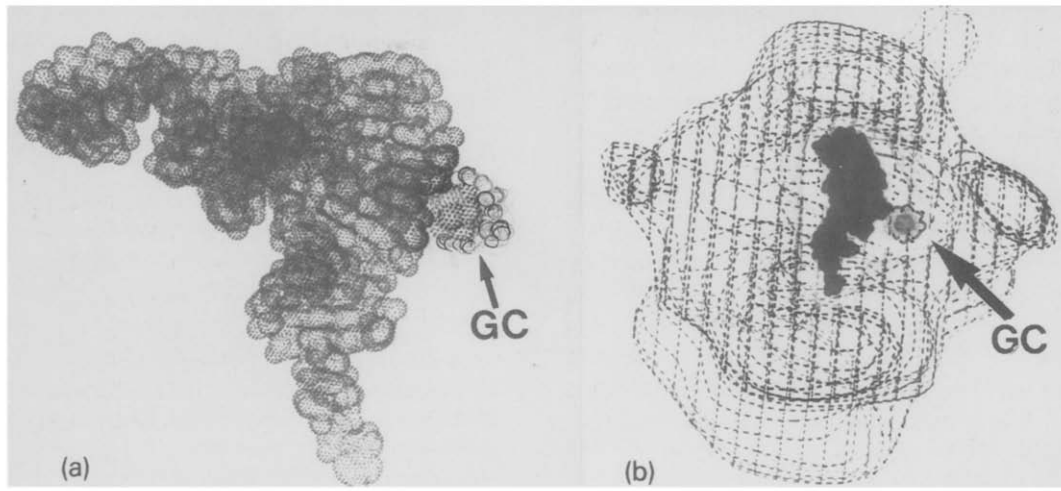

Fig. 4. (a) Computer simulation of a tRNa molecule [16] to which an undecagold cluster (coordinates taken from ref. [15]) was attached at position 47. (b) Simulation of a possible mode of interaction of the "model-built" tRNA-GC molecule with the ribosome, according to the guidelines described in ref. [8].

ments, using the coordinates of a typical tRNA molecule [16] and of a very similar gold cluster, in which fluorine atoms replace the CN moieties which are directly bound to three gold atoms of the core of the cluster [15]. As seen in fig. 4, there is at least one conformation in which a $tRNA^{phe}$–GC molecule fits into the intersubunit space with no steric hindrance.

In conclusion, we have shown that it is possible to bind an undecagold cluster specifically and quantitatively to ribosomal particles, that the modified particles can be crystallized isomorphously with native ones and that crystallographic data can be collected from these crystals. We also showed that it is possible to quantitatively modify $tRNA^{phe}$ molecules with the undecagold cluster, without impairing the functional properties of aminoacylation by its cognate synthetase and of binding to 30S ribosomal subunits.

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