Novel Procedures for Derivatization of Ribosomes for Crystallographic Studies*

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Undecagold and tetrairidium clusters have been used for the preparation of heavy-metal derivatives of ribosomal particles, necessary for the evaluation of phases in the x-ray structure determination of these large particles. To obtain specific binding, monofunctional reagents of the clusters were prepared and were covalently bound to free sulfhydryl groups on the surface of the ribosome. In addition, a mutant of *Bacillus stearothermophilus* which lacks one ribosomal protein (BL11) was grown. The heavy-atom clusters were covalently bound to isolated protein BL11, and the modified protein was consequently reconstituted into the mutated ribosomal subunits. Crystallographic data have been collected from crystals of native particles, from the mutated ones, and from the iridium- and gold-derivatized subunits. All these crystal forms are isomorphous within the experimental error.

To advance our understanding of the molecular mechanism of protein biosynthesis, we undertook crystallographic studies of intact ribosomal particles (Yonath *et al.*, 1986a, 1987, 1988a, 1988b; Makowski *et al.*, 1987; Yonath and Wittmann, 1988; Müssig *et al.*, 1989). The enormous size of ribosomes (molecular weights of 2,300,000, 850,000, and 1,450,000 for bacterial ribosomes and their small (30 S) and large (50 S) subunits, respectively dictates the use of extremely heavy and dense groups for phasing. Clusters of heavy metals which contain a core of several metal atoms linked directly to one another comprise a system of especially high electron density and seem to be most suitable for derivatizing the ribosomal particles. We are using several such clusters, among them an undecagold cluster (GC),[1] a tetrairidium cluster (IC) (both described below), and tetrakis(acetoxymercuri)methane. The last was the key heavy-atom compound, introduced by soaking, in the structure determination of the nucleosome and of

the membrane reaction center (Richmond *et al.*, 1984; Deisenhofer *et al.*, 1984).

Ribosomal particles are extremely complex, and their surfaces provide a variety of potential binding sites for the metal clusters. Therefore, soaking of crystals of ribosomal particles in solutions of heavy-atom clusters may result in attachment of the clusters to multiple sites on the ribosomes and thus may complicate phase determination or make it impossible. Hence, in parallel with our soaking experiments, we have attempted to obtain usable derivatives by binding the heavy-metal clusters to specific sites. This goal may be achieved by direct interaction of a heavy-atom cluster with chemically active groups such as —SH on the surface of the ribosome prior to crystallization. Alternatively, the cluster can be bound covalently to isolated ribosomal components that are consequently reconstituted into the ribosome. Still another way is to attach clusters to tailor-made carriers that bind to one or a few specific locations on ribosomes.

We have used chemical procedures for obtaining particles in which a few proteins are missing (Gewitz *et al.*, 1987). One of these proteins is BL11, which contains one sulfhydryl group accessible on the surface of the 50 S particle of *Bacillus stearothermophilus* and is suitable for binding the clusters (Yonath *et al.*, 1987, 1988b). In parallel, a mutant of the same bacterium, in which this particular ribosomal protein (BL11) is missing from the large subunit, has been grown. Incorporation of BL11 into the mutant particles resulted in regaining the original activity of wild-type particles (Gewitz *et al.*, 1987). The mutated subunits could be crystallized in two and three dimensions under the same conditions as the particles from the wild type. Moreover, the two crystal forms obtained from the mutated subunits are isomorphous to the corresponding forms of the same particles from the wild-type bacteria (Yonath *et al.*, 1986a, 1986b; Müssig *et al.*, 1989). This indicates that for both crystal forms, the missing protein, BL11, is not involved in the crystal forces networks. Therefore, modifying BL11 with heavy-atom clusters was expected not to interfere with crystal packing and isomorphism, thus providing a route to suitable derivatized ribosomal particles.

In this paper, we describe the procedures used by us for obtaining 50 S ribosomal subunits to which heavy-atom clusters are specifically bound. We believe that the use and the extension of our current methods will lead to the production of an extremely useful tool for extracting phases and for the location of specific sites on the ribosomes.

EXPERIMENTAL PROCEDURES

Ribosomes and Crystallization—Ribosomes and their subunits were prepared, and their integrity and activity were checked as described (Yonath and Wittmann, 1988). The mutant of *B. stearothermophilus* (TST), lacking protein BL11, was obtained by growing the cells in the presence of thiostrepton. The core 50 S particles were depleted

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[1] The abbreviations used are: GC, undecagold cluster; IC, tetrairidium cluster; NEM, *N*-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; MPH, *β*-maleimidopropionic acid *N*-hydroxysuccinimide ester; MCH, *ε*-maleimidocaproic acid *N*-hydroxysuccinimide ester; HPLC, high performance liquid chromatography.
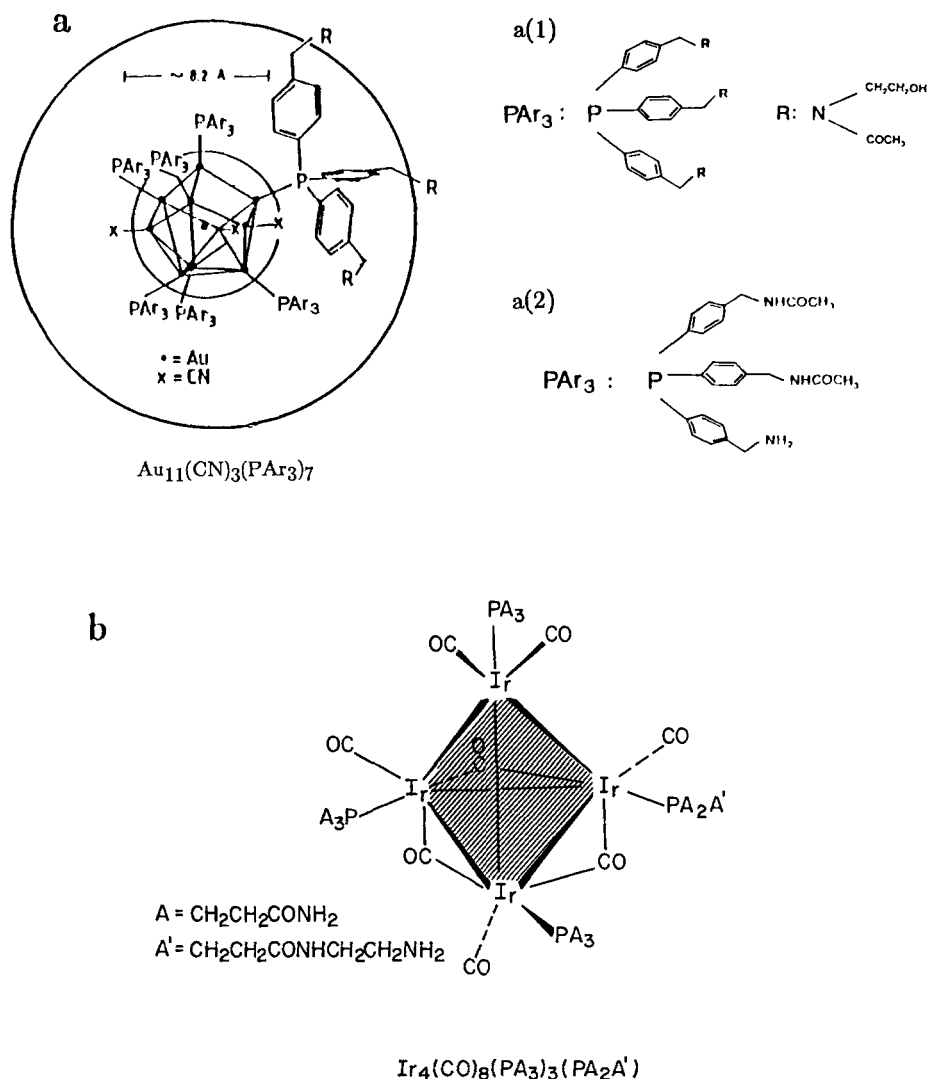
FIG. 1. *a*, schematic presentation of the undecagold cluster, depicting the gold core of 8.2 Å diameter and the arrangement of the ligands around it. The scheme is based on the crystal structure of a similar undecagold cluster (Bellon *et al.*, 1972; Wall *et al.*, 1982). *b*, schematic presentation of the tetrairidium cluster, depicting the tetrahedral arrangement of the four metal atoms. The scheme is based on the structure of similar compounds (Wilkes, 1966). *IR*, iridium; *PA*, similar to PAr₃ in *a*, but the aromatic rings are aliphalic side chains, with A, A' defined in the figure.

of six proteins (BL1, BL6, BL10, BL11, BL12, and BL16) as described by Gewitz *et al.* (1987). The procedures for growing three-dimensional crystals and two-dimensional sheets were described in detail (Yonath *et al.*, 1986a, 1987, 1988a, 1988b; Arad *et al.*, 1984, 1987; Glotz *et al.*, 1987; Makowski *et al.*, 1987; Yonath and Wittmann, 1988; Müssig *et al.*, 1989).

Determination of Exposed Sulfhydryl Groups—^{14}C-Labeled *N*-ethylmaleimide (NEM) was reacted with ribosomal particles to determine the accessibility of sulfhydryl groups on the 50 S subunits from *B. stearothermophilus* and from *Halobacterium marismortui*. The proteins that bound *N*-ethylmaleimide were identified by locating the radioactivity in two-dimensional polyacrylamide gel electrophoresis (PAGE) of the ribosomal proteins. Two-dimensional PAGE experiments were conducted as described by Kaltschmidt and Wittmann (1970).

Preparation of Activated Gold and Iridium Clusters—The undecagold cluster was prepared by a modification of the method described by Bartlett *et al.* (1978). We used the cluster with seven tris-[*p*-(aminomethyl)phenyl]phosphine groups (Fig. 1*a*, cluster with all *R* = NH₂), and in addition, we prepared another modification of the same cluster in which hydroxyethyl and acetyl groups were bound to all 21 amino nitrogen atoms of tris-[*p*-(aminomethyl)phenyl]phosphine (Fig. 1*a*, cluster with all *R* groups as in *a(1)*). This new compound is soluble in water even at very high salt concentration (3.5 M). Details of the synthesis are described elsewhere (Jahn, 1989b). This *N*-peracetylated cluster was transformed to a monoamino cluster (Fig. 1*a*, cluster with six PAr₃ as in *a(1)* and one PAr₃ as in *a(2)*) by ligand exchange (Jahn, 1989b). For all preparative experiments, the concentration of the gold cluster was estimated based on the extinction coefficient of a similar undecagold cluster, as determined by Reardon

and Frey (1984). A water-soluble tetrairidium cluster (Fig. 1*b*) was prepared from dodecacarbonyl tetrairidium by exchange of four carbonyl groups by tris-(2-ethoxycarbonylethyl)phosphine. After partial reaction with ammonia, a monoester-undecaamide was isolated. The latter was reacted with ethylenediamine to yield a monoamino tetrairidium cluster (Jahn, 1989a).

A bifunctional reagent was attached to the free amino group on the clusters via an *N*-hydroxysuccinimide ester, introducing a maleimido group for covalent binding to ribosomal particles through accessible sulfhydryl groups. The maleimido derivatives were prepared by reacting the clusters with β-maleimidopropionic acid-*N*-hydroxysuccinimide ester (MPH) to yield clusters with the group -COCH₂CH₂-(*N*-maleimide) bound to the amino-nitrogen atom. In several experiments, the maleimido group was attached through a longer arm, using ε-maleimidocaproic acid *N*-hydroxysuccinimide ester (MCH). The detailed procedure is described elsewhere.[2] The products were used immediately after preparation or kept for no longer than 2 days at −80 °C.

Binding of NEM and of the Heavy Atom Clusters to Protein BL11—NEM, GC, and IC were covalently attached to protein BL11 to produce BL11(NEM), BL11(GC), and BL11(IC). Protein BL11 was isolated from the wild-type ribosomes as described by Gewitz *et al.* (1987). The reaction was carried out under denaturation conditions (in solution A: 6 M urea, 70 mM sodium chloride, and 15 mM acetate buffer, pH 5.4). In binding NEM, 100-fold excess of the reagent was added, and the reaction mixture was kept at 4 °C for 40 h. The mixture was treated with 20 μl of 0.5 M dithiothreitol, and the excess NEM was removed by dialysis. The extent of binding was estimated

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[2] W. Jahn and S. Weinstein, unpublished results.

by measuring the radioactivity associated with the protein. For binding the clusters, BL11 in solution A was added to the dry maleimido derivatives of the clusters, to yield a 15-fold M excess of the clusters to the protein. The reaction mixture was kept at 4 °C for 40 h and then treated with 20 μl of 0.5 M dithiothreitol. A small aliquot was analyzed by 15% SDS-PAGE and by HPLC to determine the extent of reaction. Unreacted clusters and traces of BL11 were removed by gel filtration on Sephadex G-75 at 4 °C in buffer R, described below. The fractions containing the derivatized protein were identified by SDS-gel electrophoresis. BL11(GC) was heated (in buffer R) for a few hours or left at 4 °C for a few days, and its integrity and stability were determined by HPLC or by gel electrophoresis.

Reconstitution of Derivatized BL11 into Mutated 50 S Subunits—Derivatized BL11 in the urea solution was dialyzed against reconstitution buffer R of the composition: 20 mM Tris buffer, pH 7.5, 20 mM magnesium acetate, 0.4 M ammonium chloride, 0.2 mM EDTA, and 4 mM mercaptoethanol. The reconstitution was performed as described by Gewitz *et al.* (1987), the protein to 50 S subunit ratio being 1.5 and the reaction time 50 min. The reaction mixture was separated on a Sephadex G-75 column at 4 °C in a buffer chosen according to the intended further use of the reconstituted subunits (*e.g.* crystallization and two-dimensional PAGE analysis).

Direct Binding of NEM and of the Heavy-metal Clusters to Ribosomal Particles—Ribosomal particles were dialyzed to remove all traces of mercaptoethanol and to obtain the desired pH for reaction. The dialysis buffers were those in which the ribosomes were originally prepared and stored, except for the omission of mercaptoethanol. When 5.4 was the derived pH for reaction, sodium acetate was used. In the case of *H. marismortui*, the concentrations of potassium and magnesium chloride in the buffers were also varied. NEM, dissolved in the same buffer as used for the reaction, was added in about 100-fold excess, and the mixture was left to react at 4 °C for 48 h. The reaction was stopped with dithiothreitol and dialyzed to remove excess NEM. When binding the clusters, they were added in about 150-fold M excess. The mixture was left to react for 48 h at 4 °C and stopped by dithiothreitol. The ribosomal subunits were separated from their reaction mixture by gel filtration on a Sephadex G-75 column in the appropriate buffer.

Neutron Activation Analysis of Gold and Iridium—The 50 S subunits that had been derivatized with heavy-metal clusters were irradiated in the core of the nuclear reactor in the Soreq Nuclear Research Center, Israel Atomic Energy Commission, for 105 min in a flux of 2.10^{13} ns^{-1} cm^{-2}. After 4 days, needed for decay of short lived isotopes, the samples were measured in a γ-spectrometer (100 ml of Ge(Li)) for 10 min.

Crystallographic Data—Crystallographic data were collected at 85 K on film, using the rotation camera, with synchrotron radiation (λ = 1–1.5 Å), at crystal to film distances of 180–600 mm.

RESULTS AND DISCUSSION

We have used two clusters for specific binding to 50 S subunits: GC and IC with molecular masses of 6500 and 2000 daltons, respectively. According to Crick and Magdoff (1956) and assuming full occupancy in one binding site, the average change in diffraction intensities induced by binding of the undecagold cluster to the 50 S subunit is expected to be 16% and that of the iridium 10%. Hence, provided these requirements are fulfilled, the clusters are likely to be suitable for extracting phases. The diameter of the heavy atom core of the GC is 8.2 Å (Bellon *et al.*, 1972) and that of the iridium is about 5 Å (Wilkes, 1966). Thus, the IC is suitable for studies at higher resolution than the GC. The densities of the electrons around the metal cores are 3.0 and 4.7 electrons/Å^3 for the GC and IC, respectively. These values show that although the IC is relatively light, it might bring about significant intensity changes.

Crystals of 30 S ribosomal subunits from *Thermus thermophilus* as well as 50 S from *H. marismortui* and from *B. stearothermophilus* (Yonath *et al.*, 1986a, 1986b, 1988a, 1988b; Müssig *et al.*, 1989), obtained in our laboratory, were soaked in solutions of the gold cluster in which all the phosphine groups carried free benzyl-amino groups (Fig. 1a, cluster with $R = NH_2$) and in solutions of the iridium cluster (Fig. 1b).

For each of these crystal types, the native and the soaked crystals show isomorphous unit cell constants (at 12–18 Å).

The clusters were also prepared as monofunctional reagents for binding to accessible —SH groups. These were used to label the ribosomal particles in two ways. One way was to react the activated clusters with the intact particles, and the second was to react them with an isolated ribosomal protein that was subsequently incorporated into the ribosomal subunit. Free sulfhydryls on the surface of the 50 S subunit were located by reaction with radioactive NEM followed by polyacrylamide gel electrophoresis of the ribosomal proteins. Those proteins that had reacted with NEM were identified by the radioactivity in their corresponding spots. We were able to define conditions under which two proteins in the 50 S subunits from *B. stearothermophilus* were bound to NEM, whereas in the 50 S subunits from *H. marismortui*, most of the radioactivity was associated with one protein.

Since the gold cluster is rather bulky, its accessibility was varied by attaching to it the maleimido group through straight chain molecules of differing lengths (*i.e.*, MPH and MCH). In addition, the —SH groups on the surface of the 50 S particle were also extended by attaching to them a long chain residue (details will be published elsewhere). ^{14}C-labeling of the gold cluster as well as neutron activation analysis of both the gold and the iridium clusters enabled us to determine the extent of binding of the cluster to the particles. The results of both analytical methods show that a spacer of minimum length of about 10 Å between the —SH group of a ribosomal protein and the nitrogen atom of the cluster is needed to allow binding of the gold cluster directly to the 50 S particle.

We had to refine a few parameters in order to obtain optimal binding of the clusters to the ribosomes. To limit the reaction of the maleimido group to exposed —SH groups of cysteine on the surface of the ribosomal subunits, the binding was conducted at pH 5.4, which is the pH at which the 50 S subunits of *H. marismortui* are crystallized without impairing their activity. We found that we could bind one equivalent (± 6%, calculated by the absorbance of 260 nm) of GC to the 50 S subunits from *H. marismortui*, but there is no indication yet for single site binding. The yield of the direct binding of gold cluster to 50 S particles from *B. stearothermophilus* was only 0.3 equivalents cluster/ribosomal particle.[2]

The second and more successful approach was to take advantage of the TST mutant of *B. stearothermophilus*. As mentioned above, the large ribosomal subunits of this mutant lack protein BL11. This protein was shown not to be involved in the interparticle contacts within crystals of the wild-type subunits, grown under two different conditions (Yonath *et al.*, 1986b; Glotz *et al.*, 1987; Müssig *et al.*, 1989).

BL11 has one sulfhydryl group that is accessible to NEM in the intact 50 S particles. However, binding of NEM to BL11, when isolated from the ribosome, was possible only under denaturing conditions (6 M urea). It appears therefore that the conformation of the isolated protein is different from its conformation *in situ*. At 6 M urea, we could also obtain very efficient binding of the two heavy-metal clusters to the isolated protein. The reaction of BL11 with the iridium cluster was quantitative, and with the gold cluster it went almost to completion; only trace amounts of the unreacted protein could be observed in the SDS gels. It is noteworthy that binding of the clusters changed the mobility of the protein in the gels, and the corresponding bands were found in positions expected for proteins whose molecular weights are the sum of those of BL11 and of the clusters (Fig. 2).

Protein BL11 may serve as a "model sulfhydryl protein" for estimating the level of reactivity of the —SH group as
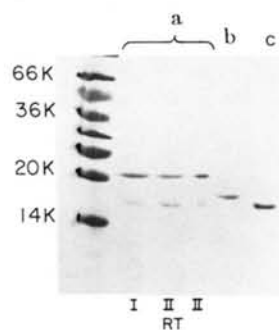
FIG. 2. **SDS-PAGE of BL11(GC) (*a*), BL11(IC) (*b*), and BL11 (*c*).** *I* and *II* indicate derivatization of BL11 with MPH and MCH, respectively. The reactions were carried out at 4 °C unless marked by *RT* (room temperature).

well as the stability of the bond between the —SH and the clusters. As discussed above, this sulfhydryl group was found to be fully reactive (Fig. 2). The products of the reaction between the —Sh (on BL11) and the clusters BL11(GC) and BL11(IC) were found to be most stable. For example, BL11(GC) was heated for 3 h at 56 °C in buffer R, which contains 4 mM mercaptoethanol, and in a parallel experiment, in the same buffer without the mercaptoethanol. In the latter case, there was no detectable disintegration of BL11(GC). Moreover, even when using buffer R, in the presence of an excess of mercaptoethanol, which competes with the ribosomes for the cluster (by ligand exchange), only 4–4.5% (after 1 h) and 9–9.5% (after 3 h) of BL11 were found to be devoided of the cluster. No further disintegration could be detected even after a few days of 4 °C, and the modified protein could be incorporated into mutated core particles to give almost fully (92–98%) derivatized 50 S subunits.

Derivatized BL11 was reconstituted with the 50 S subunits of the TST mutant to form modified 50 S particles. The reconstitution of BL11(NEM) was found to be quantitative, as could be established by determining the radioactivity associated with a known amount of subunits. The reconstitution of the protein bound to heavy-metal clusters was determined by two methods: neutron activation analysis of the heavy atoms, and two-dimensional PAGE of proteins that had been selectively detached from the ribosome (Gewitz *et al.*, 1987). All the samples of reconstituted particles with gold and with iridium clusters tested by neutron activation analysis demonstrated that one cluster molecule ±5%, calculated by the absorbance at 260 nm) was bound to each 50 S subunit. The two-dimensional PAGE showed that the reconstituted particles contained BL11 with the cluster attached to them. In the gel of proteins from the reconstituted particles with BL11(IC), there appears a new spot that is somewhat different in color, a little more diffuse, and slightly slower migrating than the native BL11 (Fig. 3). Since there is no BL11 present in the particles from the mutant, this new spot corresponds to the reconstituted BL11(IC). The gels from 50 S particles containing reconstituted BL11(GC) are somewhat different (Fig. 3). Here, we could clearly locate two new spots: one corresponding in location to BL11, and an additional tailing spot above it. It seems that BL11(GC) decomposed partly, either during the process of stripping the proteins from 50 S subunits or during the lengthy procedure of running the two-dimensional gel. Indeed, in parallel control experiments, it was shown that isolated BL11(GC) submitted to the procedure used for stripping the proteins from 50 S followed by electrophoresis and BL11(GC) submitted only to electrophoresis gave the same pattern: namely, a spot corresponding to native BL11 and an additional tailing one that corresponds to BL11(GC).
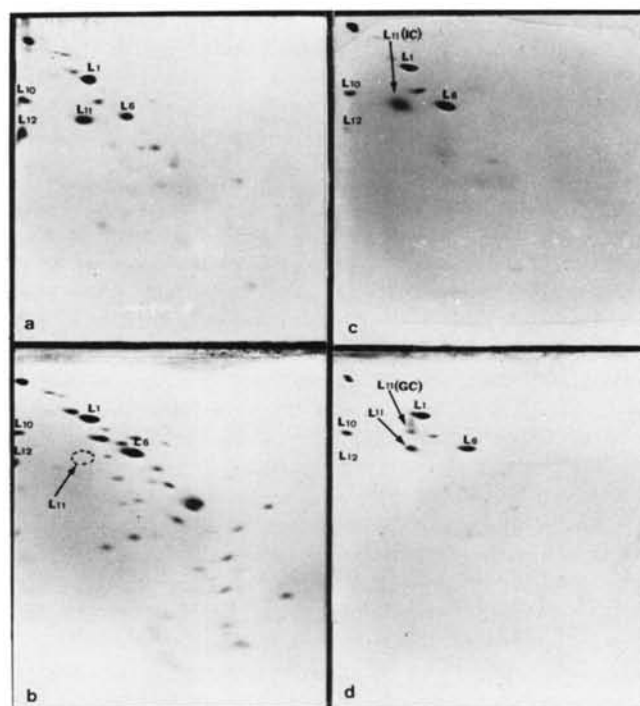

FIG. 3. **Two-dimensional PAGE of ribosomal proteins split from 50 S subunits of *B. stearothermophilus* (*a*), 50 S subunits from the mutated ribosomes (TST) (the expected location of BL11 is marked by a *dashed line circle* (*b*), reconstituted subunits obtained from BL11(GC) and 50 S of the TST mutant (*c*), and reconstituted 50 S subunits obtained from BL11(IC) and 50 S of the TST mutant (*d*).**
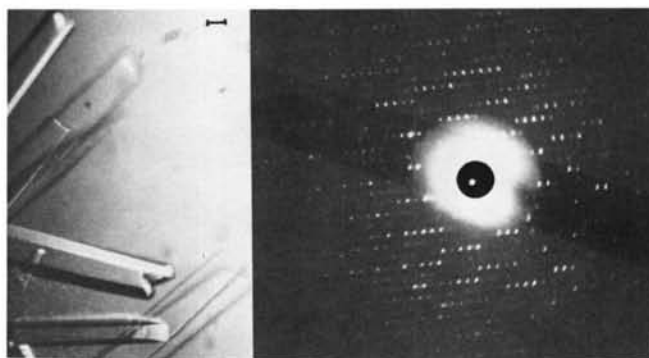

FIG. 4. **Crystals of reconstituted subunits obtained from BL11(IC) and 50 S subunits (grown as in Müssig *et al.*, 1989) of the TST mutant and their diffraction pattern.** *Bar*, 0.2 mm.

It is interesting that the 50 S subunits, after the incorporation of the derivatized or the native BL11, are biologically active and could be crystallized in two and three dimensions (Müssig *et al.*, 1989) under the same conditions as used for crystallization of the 50 S particles from the wild type of *B. stearothermophilus* (Fig. 4). Furthermore, neutron activation analysis showed that the dissolved crystals of the mutated 50 S subunits reconstituted with BL11(GC) contained the gold cluster. Preliminary crystallographic experiments performed with an intense synchrotron radiation beam at 85 K showed that the crystals of the 50 S subunits containing the gold or the iridium clusters are, within the experimental error (±0.8%), isomorphous to the native ones. Thus, the way for subsequent crystallographic analysis has been paved.

Furthermore, the R merge (I), computed for comparing between the derivatized *versus* the native crystals, shows

significant changes in intensities. A typical value of 4.5% was obtained for some 2500 reflections (about 28% of the set) which were merged together, whereas each individual set has an R value of 7–8% (*e.g.* 6.9% for 7500 of the gold-labeled crystals, and 7.8% for 4500 reflections of the native).

CONCLUDING REMARKS

We have shown that specific covalent binding of heavy-atom clusters to ribosomal particles is possible, and we have developed a procedure that enables us to label ribosomal particles without introducing major changes in their integrity, conformation, and biological activity.

The compounds used by us may also be attached to tailor-made carriers that bind to one or a few specific sites on ribosomes. Appropriate carriers may be antibiotics (Nierhaus and Wittmann, 1980) or DNA oligomers complementary to exposed single-stranded rRNA regions (Hill *et al.*, 1986). Since most of the interactions of these materials are characterized biochemically, the crystallographic location of the heavy-atom compounds will not be used for phase determination only but will also reveal the location of specific sites on the ribosome. Of special interest in this context is tRNA. Crystals and two-dimensional sheets of 50 S particles to which a molecule of tRNA as well as a short nascent polypeptide chain are bound, have already been obtained (Gewitz *et al.*, 1988). Preliminary crystallographic experiments show that the crystals are suitable for analysis by x-rays (Müssig *et al.*, 1989). A natural extension of this research line will be to bind the clusters to the tRNA molecule or to an amino acid moiety on an acylated tRNA. The modified amino acid is expected to be incorporated into the nascent protein. These derivatives may be used to obtain phase information, to locate the exact site of tRNA binding, and to reveal the path taken by the growing polypeptide chain within the ribosomal particle.

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