Immunoelectron microscopy of ribosomes carrying a fluorescence label in a defined position

Location of proteins S17 and L6 in the ribosome of *Escherichia coli*

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By coupling fluorescein to a defined amino acid of a single ribosomal protein and incorporating this protein into the ribosome, we have obtained ribosomes labelled at a single, defined position. A fluorescein-specific antibody preparation was used to locate the fluorescein residues bound to the two cysteines at positions 58 and 63 of protein S17 and to the cysteine at position 86 of protein L6. This study demonstrates the advantages which accrue from the combination of electron microscopy and fluorimetry.

Ribosomal proteins S17 and L6 *Immunoelectron microscopy* *Antibody* *Fluorescence* *Anisotropy* *Quenching*

1. INTRODUCTION

Immunoelectron microscopy (i.e., the localisation of antigenic determinants on macromolecular assemblies by electron microscopy of their complexes with antibodies) and energy transfer between two fluorophores have become standard techniques for investigation of the structure of the ribosome [1–6]. The combination of these two techniques provides the unique possibility of comparing distances between defined points on ribosomes determined in one case by microscopy of dehydrated particles and in the other case by measurements in solution. In addition, the well-known problems due to antibody contamination are eliminated from the beginning when a single fluorophore is the only reactive antigen.

We have modified proteins S17 and L6 with 5-iodoacetamidofluorescein and, following reconstitution of the modified proteins into intact ribosomal subunits, mapped their position with antibodies specific for fluorescein.

Abbreviations: Fl–S17, Fl–L6, fluorescein conjugates of proteins S17 and L6, respectively

2. MATERIALS AND METHODS

2.1. *Labelling of 30 S and 50 S subunits*

70 S ribosomes were isolated [7] and used for the preparation of subunits [8] and of purified proteins [9]. Proteins S17 and L6 were labelled with 5-iodoacetamidofluorescein and the products were characterized as in [10–12]. Whereas L6 was labelled at its single cysteine in position 86 [10], S17 has two cysteines at positions 58 and 63; no attempt was made to distinguish chemically between these (cf. [11]). Subunits were reconstituted with fluorescence-labelled proteins and characterized as in [12,13]; the reconstituted subunits had normal Svedberg values and protein content and possessed about 50% of the poly(Phe)-synthetic activity of native subunits, irrespective of the presence of the label. The 90-min incubation at 50°C, needed to activate the 50 S subunits [14], was omitted as it caused loss of label, and no gross structural dif-

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ference between incubated and non-incubated subunits was observed [12].

2.2. *Sucrose density gradient analysis*

This was carried out in NH_4Cl 100 mM, $Mg(OAc)_2$ 5 mM, Tris–HCl (pH 7.5) 50 mM, with 10–30% sucrose for 16 h in an SW40 rotor at 23000 rev./min (30 S) or 19000 rev./min (50 S). As a control, antibodies from pre-immune serum were tested; they reacted neither with native ribosomal subunits nor with reconstituted subunits containing a fluorescein-labelled protein. Likewise, anti-fluorescein failed to react with subunits containing no fluorescein label.

2.3. *Fluorimetry*

Fluorescence intensity and anisotropy were measured [10] with an SLM 8000 DS spectrofluorimeter, employing vertically polarised excitation at 495 nm and polarised emission scanned and averaged between 506 and 528 nm (bandwidths 8 nm). In each case a parallel sample containing subunits without antibody was used as a blank, with subtraction of corresponding values. The effect of rotation of the polarised light by the sucrose was negligible.

2.4. *Electron microscopy*

Samples for electron microscopy were taken directly from the sucrose gradients and prepared using the double-layer carbon technique [15]. The specimens were negatively contrasted with 0.5% uranyl acetate and photographs were taken at an instrumental magnification of 110000 in a Philips EM 301 operated at 80 kV.

3. RESULTS

3.1. *Antibody binding*

Fig.1 shows the sucrose density gradient profiles for subunits containing Fl–S17 and Fl–L6. The absorbance A_{254} and the fluorescence intensity are displayed. The figure illustrates clearly the effects of adding anti-fluorescein to ribosomal subunits containing an exposed fluorescence-labelled protein: a fluorescing dimer peak appears and there is a substantial loss of overall fluorescence intensity. The strong quenching demonstrates that the anti-fluorescein is binding to all (or, at least, to most)

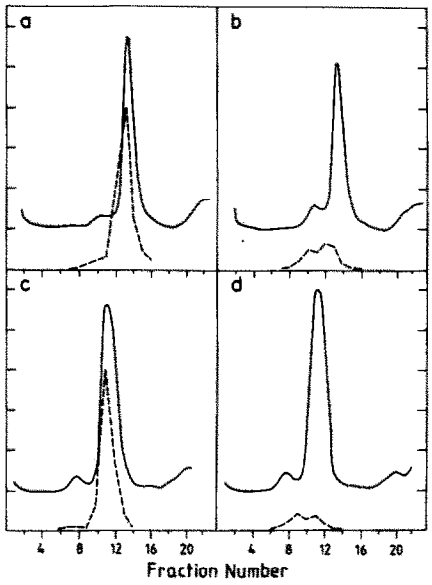

Fig.1. Sucrose density gradient centrifugation of 30 S subunits containing Fl–S17 (a,b) and of 50 S subunits containing Fl–L6 (c,d). (——) A_{254}; (– – –) fluorescence intensity; direction of sedimentation, right to left. Scales are arbitrary, with absorption zero raised by 1 scale division for clarity: (a,c) no antibody; (b,d) after incubation with a saturating amount of anti-fluorescein. N.B. The slight discrepancy between the positions of the A_{254} and fluorescence maxima for monomers arises because the A_{254} was measured continuously and the fluorescence was measured for each, separate fraction.

of the labelled subunits and not merely to a small number of aberrants.

The fluorescence anisotropies were also used as a test of binding. For example, 30 S subunits containing Fl–S17 (fig.1a, fraction no.13) had an anisotropy value of 0.245, which rose to 0.290 when antibody was present (fig.1b, fraction no.13). This rise reflects the immobilisation of the fluorescein group caused by the binding of anti-fluorescein. The dimeric complex has a still higher anisotropy value, 0.315 (fig.1b, fractions 9–10).

3.2. *Electron microscopy*

Fig.2 shows a general field and selected electron micrographs of Fl–S17-labelled 30 S subunits, reacted with anti-fluorescein. Altogether, 102 dimeric 30 S–IgG–30 S and 44 monomeric 30 S–IgG complexes were evaluated. For a

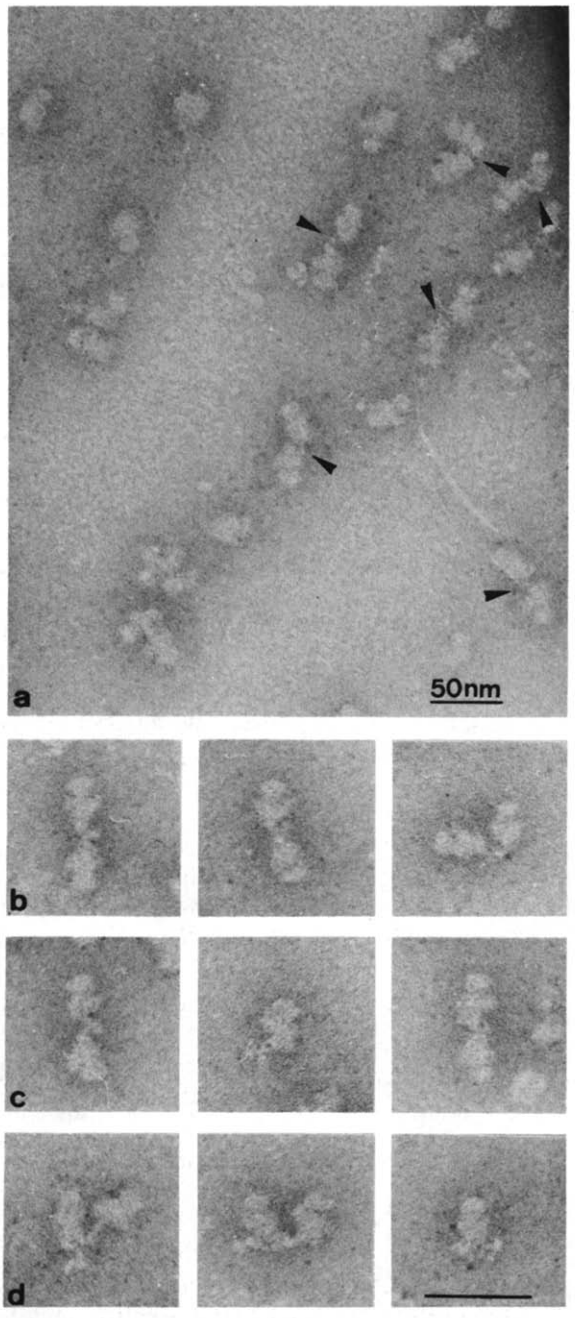

Fig.2. Electron micrographs of 30 S subunits, labelled with Fl–S17 and reacted with anti-fluorescein: (a) general field; characteristic immunocomplexes are arrowed; (b–d) selected immunocomplexes, with the 30 S subunit displayed in different projectional forms; (d) two antibodies are seen to be bound simultaneously.

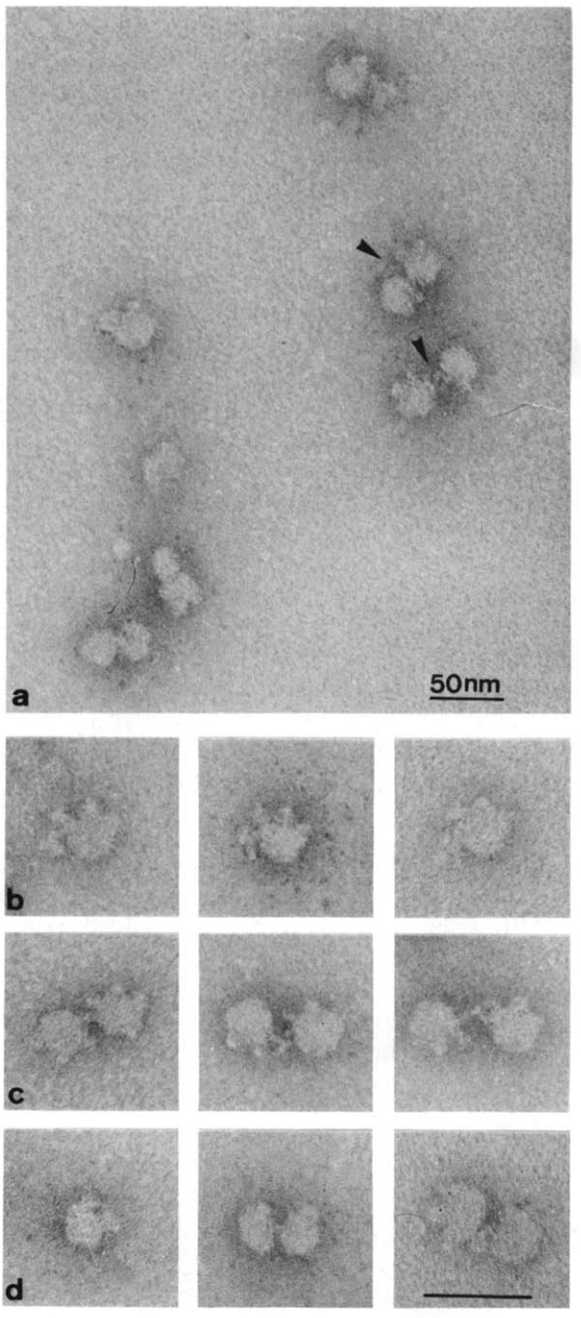

Fig.3. Electron micrographs of 50 S subunits, labelled with Fl–L6 and reacted with anti-fluorescein: (a) general field; characteristic immunocomplexes are arrowed; (b–d) selected immunocomplexes with the 50 S subunit in the crown projection (b,c) and in the kidney projection (d).

3-dimensional localisation the antibody binding site was determined on 3 different 30 S projections, which are described in detail elsewhere [13]. From the quasi-symmetric projection (fig.2b) it could be concluded that the antibody attachment site was some 30–50 Å away from the lower pole of the 30 S subunit, since the Fab arm of the connecting IgG molecule was only partly visible. In the cloven asymmetric projection, as well as in the angled asymmetric projection (fig.2c), antibody binding was at the 30 S subunit body, on the side of the large lobe, seen ~40 Å from the lower pole. A few subunits had two IgG molecules bound simultaneously (fig.2d). Few (<2%) of the 30 S–antibody complexes showed antibody binding at sites other than those described above. From these results the location of the two cysteines of protein S17 at positions 58/63 is as shown in fig.4a. This is in good agreement with the location of protein S17 on the surface of the 30 S subunit as determined with conventional antibodies [1].

50 S Subunits labelled with Fl–L6 and reacted with anti-fluorescein are shown in fig.3; 93 dimeric and 26 monomeric immunocomplexes were evaluated. In the crown form, the IgG molecule bound near the base of the L7/L12 stalk (fig.3b,c). This was especially clear from monomeric immunocomplexes (fig.3b). In the kidney projection, antibody binding was observed on the interface side of the 50 S particle, between the notch and the blunted end (fig.3d). The fluorescence label attached to protein L6 could thus be localised on the 3-dimensional 50 S model as shown in fig.4b. Again, the position corresponds to that already determined with protein-directed antibodies [1].

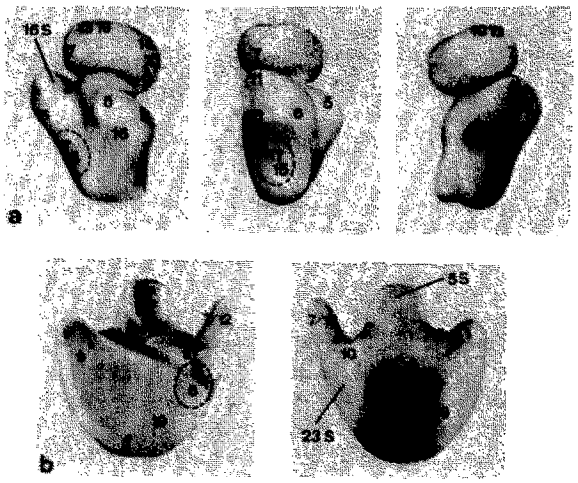

Fig.4. Three-dimensional models of the 30 S (a) and 50 S (b) subunits. The positions of proteins S17 and L6, as determined here, are outlined. The numbers give the locations of the centres of antibody binding sites for individual ribosomal proteins, determined in our laboratory with conventional antibodies [1,13,17–19]. The positions of the 3′-ends of 16 S, 5 S and 23 S rRNA [16] are also indicated.

4. DISCUSSION

The advantages of immunoelectron microscopy of extrinsic fluorescence labels are:

(i) The binding site of the antibody is specific, because only a certain protein is labelled;

(ii) Multiple binding of antibodies by a ribosome cannot occur if a ribosome carries only one labelling group. While this is necessarily true of protein L6, which has only one cysteine, protein S17 contains two cysteines very close together in the sequence. The observation of some 30 S pairs doubly bridged by two IgG molecules in close proximity to one another shows that some of the S17 molecules were labelled at both cysteines, and that both are accessible for antibody binding;

(iii) The binding can be checked by fluorimetry. The combination of quenching, anisotropy and sucrose density gradient analysis allows the separation of effects which may arise if residual amounts of free dye or free protein are present, or if the binding of the antibody causes some of the bound, labelled protein to dissociate;

(iv) Since the extrinsic hapten can be coupled to different sites, antibodies specific for a single hapten can be used for various investigations;

(v) Fluorescence quenching by the antibody and energy transfer to other incorporated fluorophores allow the results from electron microscopy to be correlated with results obtained on ribosomes and subunits in solution.

Cys-31 of protein S4 [13] and the 3′-ends of all 3 rRNAs [16] have similarly been labelled with fluorescein and the position of the fluorescein has been determined by immunoelectron microscopy (fig.4). We furthermore have preliminary data that Cys-70 of L10 and Cys-38 of L11 are located in the

same region as found with protein-specific antibodies (fig.4b). This allows us to compare distances determined by energy transfer measurements with those obtained from immunoelectron microscopy: the cysteine–cysteine distances determined for the protein pairs L6–L10, L6–L11 and L10–L11 were 50 Å, >71 Å and 67 Å, respectively, in the non-activated subunit and were somewhat shorter in the activated subunit [12]. These data are in good agreement with the location of these proteins in a single domain on the 50 S subunit (fig.4). However, the distances determined by fluorescence measurement between S4 or S17 and the 3′-end of 16 S RNA (56 Å and 62 Å, respectively [11]) are somewhat smaller than the distances estimated from immunoelectron microscopy (fig.4). It is noteworthy that the distances determined in the 70 S monosome between the 3′-ends of 16 S RNA and 5 S RNA or 23 S RNA (55 Å and 71 Å, respectively [4]) are also smaller than the distances concluded from immunoelectron microscopy [16,17]. It remains to be seen whether these differences are due to random or to systematic error.

Immunoelectron microscopy of extrinsic fluorescence labels thus provides a useful complement to, but not a replacement of, conventional immunoelectron microscopy with antibodies against protein determinants.

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