The Structure of Ribosomes as Indicated by Studies on Tetramers from Hypothermic Chick Embryos

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Ribosome tetramers induced in chick embryos by exposure to cold, and tetramers of large subunits derived from them, have been studied by electron microscopy and sucrose-density-gradient analysis. Individual ribosomes of the normal tetramer are elongated bean-shaped structures, 220-280 Å by 195 Å (1 Å = 10^{-1} nm) with a cleft in the outer edge which divides the two-dimensional image into two unequal ends. Most of the tetramers appear to attach to the surface of the electron-microscope grid by one preferred face. The subunits of the large-subunit tetramers have a round outline and no cleft. About 25% of the subunits of these tetramers have a line running radially across the particle. The dissociation of tetramers into large-subunit tetramers and small subunits has been shown to be reversible. Mixtures of these particles from sucrose-density-gradient fractions were reassociated to give a tetramer with the same sedimentation coefficient as the original tetramer and with the same structure as viewed in the electron microscope. The results indicate that the cleft is a property of the complete ribosome, and that it marks the position of the small subunit. The reversibility of the dissociation also strengthens the view that no change in the large subunit occurs during dissociation or reassociation, i.e. that the sites of interaction between ribosomes in both types of tetramer are the same. The conclusions affect the interpretation of electron-micrograph images and an anomaly in the relationship between the two types of tetramer is discussed.

A full understanding of ribosome structure at the molecular level is essential to the complete elucidation of the ribosome's function in protein synthesis.

Detailed and elegant studies such as those of Wittmann, on the ribosomal proteins (Kaltschmidt & Wittmann, 1970), of Ebel & Fellner on the RNA (Ehresman *et al.*, 1970; Fellner *et al.*, 1970), and of Nomura on the interactions between the proteins and the RNA (Mizushima & Nomura, 1970), will further advance our understanding of ribosome function only when they can be applied to a three-dimensional model of the intact particle. This point has not yet been reached.

A considerable advance in the interpretation of electron-micrograph images was made recently, when, after an exhaustive study of negatively stained preparations of rat liver ribosomes, Nonomura *et al.* (1971) derived a model for the structure of the monomer ribosomes that may be applicable to most types of ribosome. They showed that the model they proposed provides an interpretation for the observations of many other workers.

It is probable that negatively stained preparations contain more information than is currently interpretable. There is reason to believe that, if ribosomes can be arranged in ordered arrays, the techniques of image reconstruction from optical diffraction (De Rosier & Klug, 1968; Lake & Slayter, 1970) and

possibly X-ray diffraction could be applied to elucidate finer details of the structure, even though this would of necessity stop very far short of a complete structural analysis. However, the realization that details of structure, between the atomic level and that seen in electron micrographs, could be combined with the biochemical studies to advance our knowledge of the mechanisms of protein synthesis has led a number of workers to attempt to crystallize ribosomes *in vitro*.

The micro-crystalline grouping of ribosomes that has been observed *in vivo* in hypothermic chick embryos (Byers, 1967) and lizards (Taddei & Piscitelli, 1970; Taddei, 1972) suggests that crystallization *in vitro* may be possible. Consequently considerable work in recent years has been directed towards an understanding of the origin of these micro-crystals and the mechanism of their association (Barbieri *et al.*, 1970a,b; Maraldi *et al.*, 1970; Morimoto *et al.*, 1972a,b; Carey & Read, 1971).

The basic unit of the micro-crystal is a tetramer in which the ribosomes have a constant relationship to one another. These tetramers offer a unique advantage for structural studies of negatively stained material in the electron microscope. Unlike monomer ribosomes, which can adopt many attitudes relative to the grid surface, tetramers can lie in only two possible ways, and in fact only one of these seems to

be found. This simplifies the process of comparing many images.

It is now important to know whether the model of the monomer proposed by Nonomura *et al.* (1971) is supported by the morphological analysis of tetramers. In the present paper this question is examined by studying intact tetramers and those tetramers of large subunits which can be derived from them by dissociation (Carey, 1971). The reversibility of this dissociation and the re-formation of the original structure is also demonstrated.

Materials and Methods

Materials

AnalaR chemicals (Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.) were used wherever available. Embryonated eggs were obtained from Orchard Farm, Little Kingshill, Great Missenden, Bucks., U.K.

General methods

The preparation of embryos and ribosomes and the manipulation of sucrose gradients have been described (Carey & Read, 1971; Carey, 1970). Iso-kinetic sucrose-density gradients were prepared by using a pre-production version of the Isograd System (Baird and Tatlock Ltd., Chadwell Heath, Essex, U.K.). Three buffer systems, each containing 50 mM-tris, pH 7.5, have been used: TKM₁ contains in addition 25 mM-KCl and 5 mM-MgCl₂; TKM₂ contains 350 mM-KCl and 5 mM-MgCl₂; TKM₃ contains 100 mM-KCl and 10 mM-MgCl₂.

Electron microscopy

Samples for electron microscopy were taken directly from fractionated gradients. All subsequent manipulations were carried out at 4°C. A drop of the sample was placed on a carbon-coated grid for 2 min, the excess of liquid was blotted off, and the sample then fixed by immersion for 10 min in freshly prepared aq. 8% formaldehyde soln. containing the buffer used for the preparation of the ribosomes. Salts were removed from the grid by rinsing with two changes of water. Negative staining was carried out by adding a drop of aq. 2% uranyl acetate to the grid for 2 min, after which the excess of liquid was blotted and the grid dried.

Samples were viewed in a Phillips EM 300 at magnifications up to 250000. The microscope was calibrated by means of a standard lattice.

Results

Electron micrographs of negatively stained ribosome tetramers are shown in Plate 1. Tetramers are

found in the polyribosome fraction when tissues of chilled chick embryos are homogenized in 0.25 M-sucrose containing TKM₁ buffer (Bell *et al.*, 1965; Carey, 1970). The micrographs shown in Plate 1 contain much greater structural detail than has been obtained in previous studies.

Tetramer ribosomes as seen in the two-dimensional projection of negatively stained preparations are elongated, bean-shaped particles with diameters 220–280 Å and 195 Å (1 Å = 10⁻¹ nm) containing a wedge-like cleft in the outer edge. The cleft frequently continues into a line which extends across the ribosome, apparently dividing it into two unequal parts. This cleft is observed with varying clarity in most of the ribosomes of the tetramers.

The position of the cleft is illustrated in Fig. 1: it suggests that almost all tetramers are attached to the electron-microscope grid by the same surface. Thus

Fig. 1. Diagrammatic representation of the images of normal tetramers and large-subunit tetramers

The diagram illustrates the difference between the cleft and its associated line seen in a normal tetramer (a) and the line seen in large-subunit tetramers (b). Both the cleft of the normal tetramer and the line of the large-subunit tetramer are on the 'clockwise' side of the particles relative to a line bisecting the ribosomes drawn through the axis of the tetramer (central white spots). The diagram is not to scale.

the cleft of each ribosome is always 'clockwise' relative to a line, bisecting the ribosome, drawn through the axis of the tetramer. The smaller part of the particle, as viewed in two dimensions, is thus also on the 'clockwise' end of the ribosome. These structures should be compared with the illustrations shown by Nonomura *et al.* (1971), particularly their Plate 12.

Close examination of the gallery in Plate 1 indicates that the tetramers may not be symmetrical. In this gallery, the ribosome which has the deepest cleft has been placed on the left. In almost all cases the ribosome at the top of the group is different from the one at the left, in that it has a more horseshoe-shaped appearance. Indeed there is greater similarity between equivalently positioned ribosomes in different tetramers, for these two positions, than between the individual ribosomes within a single tetramer.

Structure of the large-subunit tetramer

It has previously been shown that when intact tetramers are exposed to ionic conditions sufficient to dissociate free monomers into subunits, the tetramer is dissociated into small subunits and a tetramer composed of large subunits (Carey & Read, 1971; Carey, 1971). Examination of the large-subunit tetramer provides further information regarding the nature of the cleft.

Plate 2 shows examples of the appearance of this particle as found in the isolation medium containing TKM₂ buffer. The individual large subunits appear rounded and somewhat larger than intact ribosomes of the original tetramers. They have an outline similar to that called type (i) by Nonomura *et al.* (1971). No cleft is visible. An indication of substructure of the subunit is shown by the presence on some of the particles of a line extending radially relative to the axis of the tetramer (Plate 2, arrows). This line could be caused by a shallow groove in the surface of the subunit or a narrow tube through its centre. The diagram in Fig. 1 illustrates the difference between the appearance of the line of the large-subunit tetramer and the cleft on the intact tetramer. The line on the large-subunit tetramer is approximately in the same position as the cleft and its associated line of the ribosomes of intact tetramers. The large-subunit tetramer line is clearly on the 'clockwise side' of the centre of the particle as defined above, suggesting that large-subunit tetramers also tend to attach to the grid surface preferentially by one of their faces. The line is found in 25% of the subunits of the large-subunit tetramers (200 large subunits counted on 10 fields), and may be present in one, two or three of the subunits of a large-subunit tetramer.

It was thought that the subunits of the large-subunit tetramer may be in an expanded form, owing to the

Fig. 2. Sucrose-density-gradient profiles of large-subunit tetramers (a) in TKM₂ and (b) in TKM₃ buffers. Large-subunit tetramers were prepared from ribosomes that had been prepared in TKM₁ buffer by fractionating from sucrose-density gradients in TKM₂ buffer. Samples were then diluted either with the same buffer (TKM₂) or with buffer to give TKM₃. Samples (0.8 ml) containing 0.2 E_{260} unit were layered over isokinetic sucrose gradients (11.5 ml) and centrifuged at 40000 rev./min for 85 min in a Spinco SW 41 rotor. Peak 1, free large subunits; peak 2, large-subunit tetramers. For details of buffer salt conditions, see the Materials and Methods section.

high-salt conditions necessary for their preparation, which results in poor definition and loss of the cleft when viewed in the electron microscope. Conditions

were therefore sought that would re-compact the large subunits. Raising the Mg^{2+} and lowering the K^+ concentrations were found to increase the sedimentation coefficient of the large-subunit tetramer in sucrose gradients. A variety of conditions were explored and Fig. 2 shows that lowering the KCl concentration from 250 to 100mM and raising the $MgCl_2$ concentration from 5 to 10mM increased the rate of sedimentation from 131S to 137S, as would be expected if the particles became more compact.

The large subunits in this compacted form (Plate 4b) do not seem to be significantly smaller than those of the large-subunit tetramer and in many instances the line referred to above was apparent. No clefts are visible after re-compaction, suggesting that this feature is a property of the monomer. There was no significant alteration in the general appearance of this particle compared with the original large-subunit tetramer.

Reconstruction of intact tetramers from separated subunits

The reconstruction has been achieved in two ways. In the first procedure the ionic conditions of a sample of ribosomes prepared in TKM_1 buffer were adjusted to TKM_2 buffer, which gave rise to a mixture of small and large subunits and large-subunit tetramers (Figs. 3a and 3b). The conditions were then readjusted to give TKM_3 buffer (Fig. 3c). In the latter case the distribution on a sucrose-density gradient closely resembled that of the original preparation in TKM_1 buffer, suggesting that reconstruction had occurred. Plate 3(a) shows that the reconstructed tetramers had the same appearance in the electron microscope as the original undissociated tetramer.

In the second procedure both the peaks corresponding to large-subunit tetramers and small subunits were separately isolated from sucrose-density gradients in TKM_2 buffer and then mixed before adjusting the ionic conditions to TKM_3 buffer as above. Fig. 4 shows that the large-subunit tetramers, which alone increase only slightly in sedimentation rate (Fig. 2), regain the rate of the normal tetramer when the ionic conditions were adjusted to TKM_3 buffer in the presence of small subunits. The large-subunit tetramers are slightly unstable, and during preparation they tend to separate into free large subunits, which can then recombine with small subunits to give monomers (Fig. 4b).

Plate 3(b) shows the appearance of the small subunits used in this experiment. They are elongated bean-shaped objects with a length of 220Å and maximum width of 165Å ($1\text{Å} = 10^{-1}\text{nm}$). Their appearance is very similar to that described by Nonomura *et al.* (1971) for the small subunit of rat liver. In many instances the subunit is seen to be divided into two unequal lobes, the dividing point being about one-

Fig. 3. Sucrose-density-gradient profiles of total cold-treated chick embryo ribosomes

Ribosomes prepared in TKM_1 buffer were separated on a sucrose-density gradient containing the salts of this buffer (curve a). A second sample was adjusted to TKM_2 buffer and fractionated on a density gradient containing the salts of this buffer (curve b). A sample of the ribosomes in TKM_2 buffer was then readjusted to TKM_3 buffer and fractionated similarly (curve c). In each case samples (0.2ml) containing 2.0 E_{260} units were layered over 10% isokinetic sucrose gradients (11.5ml) and centrifuged at 40000 rev./min for 80min in a Spinco SW41 rotor. Peak 1, small subunit; peak 2, large subunit; peak 3, monomer; peak 4, dimer; peak 5, large-subunit tetramer; peaks 6 and 7, complete tetramer.

EXPLANATION OF PLATE 1

Electron micrographs of negatively stained ribosome tetramers

(a) A field of normal tetramers prepared in TKM₁ buffer and separated on a sucrose-density gradient before negative staining (magnification $\times 180000$). (b-j) A gallery of selected normal tetramers (magnification $\times 310000$).

EXPLANATION OF PLATE 2

Electron micrographs of large-subunit tetramers

(a) A field of large-subunit tetramers prepared in TKM₂ buffer and separated on a sucrose-density gradient before negative staining (magnification $\times 180000$). (b-j) A gallery of selected large-subunit tetramers (magnification $\times 310000$). The arrows point to the line referred to in the text and illustrated diagrammatically in Fig. 1.

EXPLANATION OF PLATE 3

Electron micrographs of reconstructed tetramers and of small subunits

(a) Tetramers from the sucrose gradient shown in Fig. 3(c). These tetramers were prepared in TKM₁ buffer (Fig. 2a), adjusted to TKM₂ buffer (Fig. 2b) and then readjusted to TKM₃ buffer before gradient fractionation and negative staining (magnification $\times 180000$). (b) Small subunits in TKM₂ buffer from a sucrose gradient similar to that of Fig. 3(b). The arrows point to subunits in which the division into two lobes can be seen (magnification $\times 180000$).

EXPLANATION OF PLATE 4

Electron micrographs of mixtures of tetramers

(a) A mixture of large-subunit tetramers and small subunits prepared by fractionation on sucrose-density gradients in TKM₂ buffer before mixing and separating on the sucrose-density gradient illustrated in Fig. 4(a) (magnification $\times 180000$). (b) Large-subunit tetramers prepared in TKM₂ buffer and adjusted to TKM₃ buffer in the absence of small subunits (magnification $\times 180000$).

EXPLANATION OF PLATE 5

Electron micrographs of re-formed tetramers

A mixture of large-subunit tetramers and small subunits prepared by fractionation on sucrose-density gradients in TKM₂ buffer, mixed and adjusted to TKM₃ buffer before the sucrose-gradient separation illustrated in Fig. 4(b). The large-subunit tetramers recombine with small subunits to give tetramers with the appearance of the original intact group. The arrows point to the excess of small subunits (magnification $\times 180000$).

(a)

(b)

(c)

(d)

(e)

(f)

(g)

(h)

(i)

(j)

(a)

(b)

(c)

(d)

(e)

(f)

(g)

(h)

(i)

(j)

(a)

(b)

(a)

(b)

Fig. 4. Sucrose-density-gradient profiles of mixed small subunits and large-subunit tetramers (a) in TKM_2 and (b) in TKM_3 buffers

Samples of large-subunit tetramers and small subunits obtained by fractionating from a sucrose-density gradient in TKM_2 buffer were mixed and diluted, either with the same buffer or with buffer to give TKM_3 . Samples (0.8 ml) containing 0.07 E_{260} unit of small subunit and 0.13 E_{260} unit of large-subunit tetramer were layered over 15% isokinetic sucrose (11.5 ml) and centrifuged at 40000 rev./min for 95 min in a Spinco SW41 rotor. Peak 1, small subunit; peak 2, large subunit; peak 3, reconstructed monomer; peak 4, large-subunit tetramer; peak 5, reconstructed tetramer.

third of the distance along the long axis of the particle.

Plate 4(a) shows the mixture of particles, which was centrifuged in a sucrose-density gradient containing TKM_2 buffer to give the profile shown in Fig. 4(a).

The field shows large-subunit tetramers and free small subunits. When the ion concentration was adjusted to TKM_3 buffer in the absence of small subunits the tetramers had the appearance shown in Plate 4(b), that is essentially unchanged large-subunit tetramers. When the ion concentration of a mixture containing small subunits and large-subunit tetramers was adjusted to TKM_3 buffer a tetramer was re-formed that had the sedimentation coefficient of the original tetramer (Fig. 4b) and had the normal appearance found in untreated particles, as shown in Plate 5. Plate 5 also shows an excess of free small subunits in the same field.

Discussion

Nonomura *et al.* (1971) have proposed a model for the ribosome based on a detailed electron-microscope study of rat liver monomers visualized by negative staining. We must now consider how the observations of chick embryo tetramer ribosomes described here relate to this model. In particular we must ask: (i) does the cleft represent the junction of the small and large subunits?; (ii) what is the position of the small subunit relative to the cleft?; (iii) does the small subunit participate in the bonds joining the monomers of the tetramer?; (iv) is the line of the large-subunit tetramer due to residual small subunits or does it represent a structural feature of the large subunits?; (v) what is the relationship of the line of the large-subunit tetramer to a similar structural feature described by Nonomura *et al.* (1971) and what conclusions can be drawn about the location of the small subunit in the monomer from the orientation of this line?

The results in this paper suggest that the cleft in the intact tetramer is due to the presence of the small subunit, and thus may indicate its position. The cleft disappears when small subunits are dissociated and reappears when reconstruction occurs. When large-subunit tetramers are subjected to ionic conditions that permit attachment of small subunits, the cleft reappears if small subunits are available for attachment, but not if they are withheld. Changes in sedimentation coefficient, on sucrose-density gradients, demonstrate when reconstruction of complete tetramers has occurred.

The second question, concerning the position of the small subunit in relation to the cleft, is more difficult to answer. It seems most likely, however, that the small subunit will lie to the clockwise side of the cleft (as defined above) and below the large subunit. This is supported by the strong resemblance between the ribosomes of the tetramer and the tilted images of rat liver monomers reported by Nonomura *et al.* (1971). If this conclusion is correct, the long axis of each small subunit will be approximately parallel to the radius of the tetramer that bisects the monomer.

In earlier studies (Carey & Read, 1971; Carey, 1971) it was concluded that the small subunits are not essential for the bonding between the monomers of the intact tetramer, since they are free to dissociate, yielding the large-subunit tetramer. The alternative conclusion, that the small subunit of one ribosome is attached to the large subunit of another, was reached by Sabatini *et al.* (1971) from a study of electron micrographs. The reversibility of the dissociation of the small subunits reported here shows the former conclusion to be the most likely.

The answer to the fourth question is that the line of the large-subunit tetramer is not due to the presence of residual small subunits, giving a slightly altered appearance of the cleft. It has already been shown that there is no 18S RNA in the large-subunit tetramer (Carey, 1971). Results reported here show that normal and large-subunit tetramers are well separated on sucrose gradients (Fig. 2). There is no indication of heterogeneity in the large-subunit tetramer peak in TKM₂ buffer nor of any significant residuum of intact tetramers sufficient to account for the 25% of ribosomes observed to have lines. In many instances large-subunit tetramers with two or three lines were found. If these were due to undissociated small subunits the structures would have sedimentation coefficients intermediate between the normal and large-subunit tetramer and thus result in a heterogeneous profile, and this is not found. Recent experiments (N. H. Carey & E. A. Cook, unpublished work) in constructing such intermediate particles confirm this expectation. It must therefore be concluded that the line of the large-subunit tetramer is a true feature of the large subunit and is likely to be present but masked in the monomer by the small subunit.

Attempting to answer the last question posed above produces an anomaly. Nonomura *et al.* (1971) have observed a line in the rat liver large subunit, which appears to be similar to the line of the subunits of the large-subunit tetramer. They concluded that the line is due to a shallow groove in the surface, but it was difficult to observe and was found in very few electron-micrograph images. In the model for the monomer that they proposed the small subunit is attached to that part of the surface of the large subunit which contains the groove, and has its long axis at right-angles to the groove.

If this model of the monomer is used to deduce the location of the small subunits as they attach to the tetramer, their long axes would thus be at right-angles to the large-subunit tetramer line. However, since the electron micrographs do not show whether the line is on the top or bottom surface of the tetramers the small subunit could reattach to either surface. The model of Nonomura *et al.* (1971) would locate the small subunits towards the centre of the tetramer if they attach to the lower surface of the

large subunit (i.e. that nearest the specimen grid), or near the outside edge of the tetramer if they attach to the upper surface. Their long axes would thus be at right-angles to the tetramer radii, which bisect the large subunits. Such a position of the small subunits is not in accord with electron micrographs of intact or reconstructed tetramers shown here, nor does it agree with the answer to question (ii), proposed above. The anomaly is that from direct observation it seems likely that the long axes of the small subunits lie approximately parallel to the radii of the tetramer, whereas deduction from the structure of the large-subunit tetramer, with the model of Nonomura *et al.* (1971), would place them at right-angles to the radii.

This apparent anomaly may be resolved in one of three ways: (i) the line observed on the large subunits of the large-subunit tetramer is not produced by the groove described by Nonomura *et al.* (1971), or (ii) the interpretations of these authors concerning the feature which they observed in the large subunit is incorrect, or (iii) the formation of large-subunit tetramers involves a reorientation of approx. 90° of the individual large subunits.

The possibility that two different structural features could give rise to a similar observation cannot be ignored. It is possible that the line in the large-subunit tetramers described here is not a surface feature, but derived from, say, a hollow tube across the subunit. The presence of such a tube in the large subunit has been proposed to account for the protection of part of the nascent polypeptide, proximal to the tRNA, from enzyme attack *in vitro* and the translocation of the newly formed protein into the cisternae of the endoplasmic reticulum (Redman & Sabatini, 1966; Malkin & Rich, 1967; Blobel & Sabatini, 1970). The groove in the surface of the large subunit, described by Nonomura *et al.* (1971), may not be sufficiently distinct to be observed with confidence in the large-subunit tetramers.

No firm conclusion concerning the orientation of the monomer in the intact tetramer can be reached until the anomaly created by the interpretation of the large-subunit tetramer line has been resolved. This is not yet possible.

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