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The use of neutrons to study protein–RNA interactions

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Protein–RNA interactions play a key role in the structure, morphogenesis and function of various systems (viruses, ribosomes and, more generally, protein synthesis). The neutron is a powerful tool to study those interactions. Some examples, are given. For viruses, neutrons provide structural information on the two molecules where they interact. Viral proteins do not appear to be simple globular proteins. In the interactions between tRNA and aminoacyl tRNA synthetases, neutrons allow a simultaneous study of the reaction and of the structural modifications associated with that reaction, giving a hint on the role of both electrostatic and specific interactions.

Introduction

Among the interactions that play a key role in cell life, those between ribonucleic acids (RNA) and protein are specially important. RNA is found associated with many functions in gene expression in the cell, and in all cases interactions with specific proteins play a key role. This may be at several levels. First, it may be a structural role, as for instance in a ribosome, where one of the actions of the protein–RNA interaction is to establish the structure of the particle so that it will perform its functions correctly. The role of the interaction may be also directly at the functional level, as for instance in the control of the RNA messenger. Another example of this last case is that of the reactions of tRNA with various enzymes involved in protein biosynthesis, such as aminoacyl tRNA synthetase, elongation factor and formylase.

Nature of the interaction

RNA is a genuine polyelectrolyte and this is expected to play a key role in its interaction with protein. For instance, basic groups (lysine and arginine), which are positively charged, having strong electrostatic links with the negatively charged phosphates of the RNA. This type of interaction is highly salt-dependent and does not vary with the sequence of the RNA. Here we consider single-stranded RNA. Such RNA has a secondary structure formed by base pairing (cooperative hydrogen bonding) between different parts of the molecule. This structure is complex and strongly dependent upon the sequence. It has been suggested (Weidner *et al.* 1977) that a given sequence may have different secondary structures that have nearly the same energy. The RNA is expected to be folded by various forces (salt links, hydrophobic, etc.) into a three-dimensional structure, as already established for tRNA. This makes an important difference from double-stranded DNA, which has broadly speaking a conformation independent of its sequence, which modulates only the exact organization of the double helix. Single-stranded RNA should have a three-dimensional structure that depends strongly on its composition and sequence and which may be modified by external parameters. This gives the possibility of very specific and precise interactions between RNA and the proteins that are functionally associated with it.

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So far very little is known of the exact nature of those interactions. The normal approach would be to crystallize a complex between an RNA and interacting protein (for instance a complex between a tRNA and the corresponding aminoacyl tRNA synthetase). So far this has not been possible; the only crystallized systems in which RNA and proteins are in contact are viruses, and for that reason various groups (Harrison *et al.* 1978; Unge *et al.* 1979; Suck *et al.* 1978) have undertaken crystallographic studies of viruses. In the most advanced work (Harrison *et al.* 1978) a structure of tomato bushy stunt virus at 2.9 Å resolution has been obtained. This study gives much important information on protein–protein interactions, but none on protein–RNA interaction, as no density, associated with RNA, is found in the electron density map, indicating that the RNA is completely disordered. Owing to this lack of precise crystallographic information, other methods must be sought. Neutron scattering turns out to be one of the most useful.

Neutron scattering applied to nucleoprotein systems

We shall consider here only neutron scattering from solutions. This method with special references to biological applications, has been described in some detail (Jacrot 1976), and only the main points will be summarized.

In dealing with scattering from objects in solution, since only low resolution structural information can be obtained, the relevant quantity is the scattering density, which is defined by

$$\rho(r) = \frac{1}{V} \sum_{v} b_i. \tag{1}$$

The summation is done on a volume V large compared with interatomic distances, but small compared with the resolution of the data; b_i are the scattering amplitudes of all atoms included in the volume v. Indeed, as we consider objects in solution, the quantity that will come into the equations is the difference between the radial density distribution $\rho(r)$ of the object and that, ρ_s, of the solvent. In all practical cases the solvent for biological molecules is water with small amount of salts, and can be considered, from the neutron point of view, as pure water with a negative scattering density of 0.562×10^{-14} cm/Å^3†. If heavy water is substituted for ordinary water the scattering density of the solvent will rise to 6.4×10^{-14} cm/Å^3. In comparison the scattering density of a protein is about 1.8×10^{-14} cm/Å^3 and that of a nucleic acid around 4×10^{-14} cm/Å^3. The density of a protein (or a nucleic acid) increases somewhat with the amount of D_2O in the solvent. This is due to the exchange of labile protons. The contribution to the scattering curve of the two components (protein and nucleic acid) will vary with the amount of D_2O in the solvent. If the protein has a uniform density (this is never strictly true) in a solvent with some 40–42 % D_2O, only the RNA will contribute to the scattering. The reverse will be true in a solvent with 68–70 % D_2O. This simple fact is the basis of the study of nucleoproteins with neutrons. More generally, one defines the contrast of a particle as the difference between its average scattering density and that of the solvent. This contrast may be positive or negative.

† 1 Å = 10^{-10} m = 10^{-1} nm.

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The intensity at the origin

A dilute solution of N identical particles gives a scattering curve which extrapolates at zero angle to

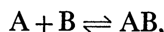
$$I(O) = (\sum b - b_s V)^2 N. \tag{2}$$

The summation is over all atoms in the particles, and V is the volume occupied by those atoms (volume from which the solvent is excluded), and is related to the specific volume $\bar{v}$ of the particle of molecular mass M by

$$V = \bar{N}^{-1} M \bar{v},$$

where $\bar{N}$ is the Avogadro number. This relation is valid only if the system is a two-phase system: molecule and solvent. If the solvent in contact with the molecule is different from the bulk solvent (2) must be modified to take this into account. If the particle is a protein, b and V are proportional to M, whereas for a concentration expressed in mass per volume N is inversely proportional to M. Thus $I(O)$ is proportional to the molecular mass of the protein. Intensities are easily calibrated by scattering from water, and so the protein molecular mass can be measured easily (Jacrot & Zaccaï 1980).

Now if one considers a reacting system made of proteins A and nucleic acid B with, for instance, the equilibrium



$$A + B \rightleftharpoons AB,$$

the solution will be composed of a mixture A, B and AB defined by the equilibrium constant of the reaction, and the intensity at the origin will be given by

$$I(O) = (\sum b_A - b_s V_A)^2 N_A + (\sum b_B - b_s V_B)^2 N_B + (\sum b_A + \sum b_B - b_s V_A - b_s V_B)^2 N_{AB}. \tag{3}$$

So if one starts with a pure solution of protein A, by adding successive quantities of nucleic acid B to A it will be possible to determine the equilibrium constant of the reaction, and more important to establish its stoichiometry. As we shall see later, this turns out to be the most reliable method to establish this stoichiometry, and its dependence on parameters such as ionic strength. The reaction can be followed in any solvent, but the stoichiometry will be better followed in H_2O where both components will contribute. If, on the other hand, one wishes to follow the behaviour of the protein moiety alone, this will be better done in a solvent where the contribution of the nucleic acid is minimized.

The radius of gyration

The intensity at very small angles is given by the Guinier approximation:

$$I(s) = I(O) \exp(-\tfrac{4}{3}\pi^2 s^2 R_G^2), \tag{4}$$

where s is the scattering vector of length $(2/\lambda) \sin \tfrac{1}{2}\theta$ where θ is the scattering angle and λ the neutron wavelength. This will give the radius of gyration, R_G, of the particle. For a particle with uniform scattering density, R_G will be independent of contrast, but for a nucleoprotein in which the density of the RNA is higher than that of the protein, R_G will vary, as shown by Stuhrmann (1974), and this variation will provide some information on the relative distribution of RNA and proteins within the particle. This has been applied to ribosomal subunits (see reviews by Koch & Stuhrmann (1979) and Serdyuk (1979)).

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If one considers again the reaction between a protein and a nucleic acid, it will be possible, by using the appropriate solvent, to follow changes of structure in one of the components during a reaction, characterized quantitatively by a change of radius of gyration.

The scattering at larger angles

Beyond the domain of validity of Guinier approximation, the scattering curve $I(s)$ is given by

$$I(s) = \left\langle \left| \int_v (\rho(\boldsymbol{r}) - \rho_s) \exp(2\pi i \boldsymbol{s} \boldsymbol{r}) \, d^3\boldsymbol{r} \right| \right\rangle, \quad (5)$$

where the bracket represents the averaging over all orientations of the particles, which in a dilute solution are always randomly orientated. It is obvious that this averaging makes impossible, in the general case, the inversion of (5) to get $\rho(\boldsymbol{r})$ and this equation can only be used to test models of structure. Here again, the measurement of $I(s)$ with various contrasts allows models of the structure of both the RNA and the protein in a nucleoprotein particle to be tested. This has been done for the 30 *S* subunit of ribosomes (Serdyuk *et al.* 1979). A model is characterized by a certain number of parameters. Information theory gives the maximum number of parameters that can be obtained from a set of curves $I(s)$ at various contrast (for the case of an infinitely accurate experiment; in practice, the limited accuracy of the data will always limit the number of parameters to a smaller value than given by theory). This point is dealt by Luzzati (1979), giving for this number of parameters

$$J = 6 + 6 s_{max} D, \quad (6)$$

where D is the largest dimension of the object and s_{max} the largest scattering vector in experimental data.

For a spherical object, the averaging over orientations disappears and (5) can be inverted. This applies to isometric icosahedral viruses, which, at least up to resolution comparable with distances between subunits, can be considered as spherical. If data are collected up to $s = (50\ \text{Å})^{-1}$ on virus of diameter 250 Å, (6) says that a maximum of 36 parameters can be obtained. Even taking into account the reduction of that number from data inaccuracy this is enough to analyse a spherical virus into four or five spherical shells (12 or 15 parameters), giving a rather detailed view of the relative organization of the RNA and the protein shell inside the virus.

Tomato bushy stunt virus (TBSV)

The method outlined above has been used to get a low-resolution structure of the virus TBSV (Chauvin *et al.* 1978). The main result of that study was to reveal an unfolding of the polypeptide chain, or a strand about ten amino acids long, in that part of the virus where most of the RNA is concentrated. This is shown in figure 1. This observation suggests the following comments.

(*a*) The inner part of the viral protein, being non-globular, may have several slightly different conformations. This gives rise to an internal disorder, which explains why this inner part of the virus is not observed by X-ray high-resolution crystallography.

(*b*) If protein–RNA interactions in the virus were purely electrostatic, this disorder would be unnecessary; on the other hand, if there are specific recognitions between an amino acid

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(or an amino acid sequence) and a nucleotide (or a nucleotide sequence), such disorder is necessary. Without that disorder the same amino acids would be found in positions related by the icosahedral symmetry, whereas the lack of periodicity in the nucleotide sequence makes difficult a folding of the RNA strand which would also bring identical nucleotides into symmetry-related positions in the virion.

(*c*) When the virus swells (J. Witz & B. Jacrot, unpublished results) at high pH in the presence of EDTA, this internal organization of the virus is preserved, showing its importance for the virus's stability.

(*d*) In another virus (brome mosaic virus), we have strong evidence that the viral protein is not globular and has a somewhat similar internal organization (M. Cuillel, B. Jacrot & M. Zulauf, unpublished results), although the protein does not go so far into the interior of the virion.

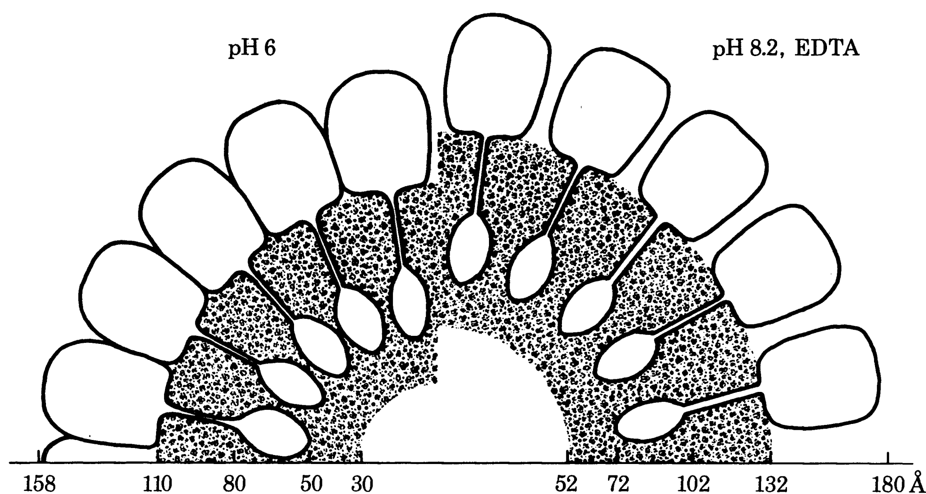

FIGURE 1. A schematic representation of the internal structure of tomato bushy stunt virus in its native state (left side) and in its swollen state (right side). The shadowed area represents regions occupied by RNA. The densities found in the swollen virus compared with those in the native one suggest that there is no reorganization during swelling but simply a displacement by 22 Å of a group of proteins (most likely trimers) with the RNA bound to it.

THE INTERACTION BETWEEN tRNA AND AMINOACYL tRNA SYNTHETASES

During protein synthesis, each tRNA must be charged with its appropriate amino acid. This is done by a group of enzymes (one for each tRNA) named aminoacyl tRNA synthetases. The reproducibility and precision of each protein synthesis is dependent on the proper recognition between each tRNA and the corresponding enzyme. This is a typical case where protein–RNA interaction plays a key role. Several systems have been investigated with neutron scattering (Dessen *et al.* 1978; Zaccaï *et al.* 1979; Zaccaï, private communication). Data were collected by titrating the enzyme with the tRNA and were analysed in terms of the neutron scattering intensity at the origin $I(0)$ and of the radius of gyration as explained above. The results obtained can be summarized in the following points.

(*a*) The stoichiometry of the reaction is easily obtained from $I(0)$ for the data in H_2O, as explained above. Figure 2 shows the interaction of $tRNA_{Asp}$ from yeast with the corresponding synthetase. The data establish that two tRNA molecules bind to the dimeric enzyme, a point that was controversial with standard biochemical methods. The neutron method is very direct.

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(*b*) The existence of electrostatic and specific interactions between the two molecules is, for instance, illustrated in figure 2. The interaction was studied in various media. The data in two of those media are shown in the figure. For molar ratio of tRNA:enzyme larger than two, the results are independent of ionic strength, whereas for smaller ratios much higher intensities are measured at low compared with high ionic strength. Similar behaviour has been observed with several systems (Zaccaï *et al.* 1979). The interpretation is unambiguous. At low ionic strength the tRNA induces the formation of aggregates of enzyme. Those aggregates are

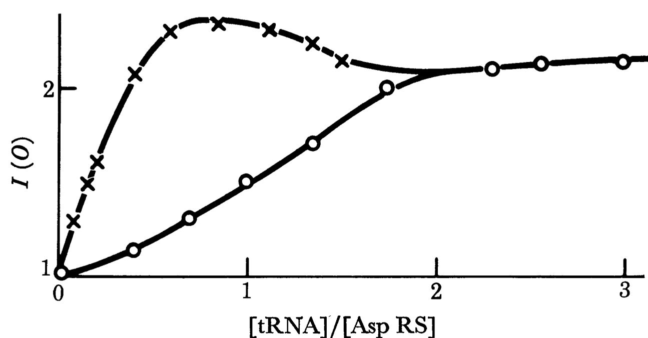

FIGURE 2. Titration of aspartic tRNA synthetase from yeast with the corresponding tRNA. On the vertical axis is shown the intensity at the origin normalized to unity for the enzyme alone. Buffer is 20 mM MES, pH 6.8 in H_2O, with 10 mM $MgCl_2$ (×) or 1.5 M $(NH_4)_2SO_4$ (○). (From R. Giege, D. Moras, J. C. Thierry and G. Zaccaï, unpublished work.)

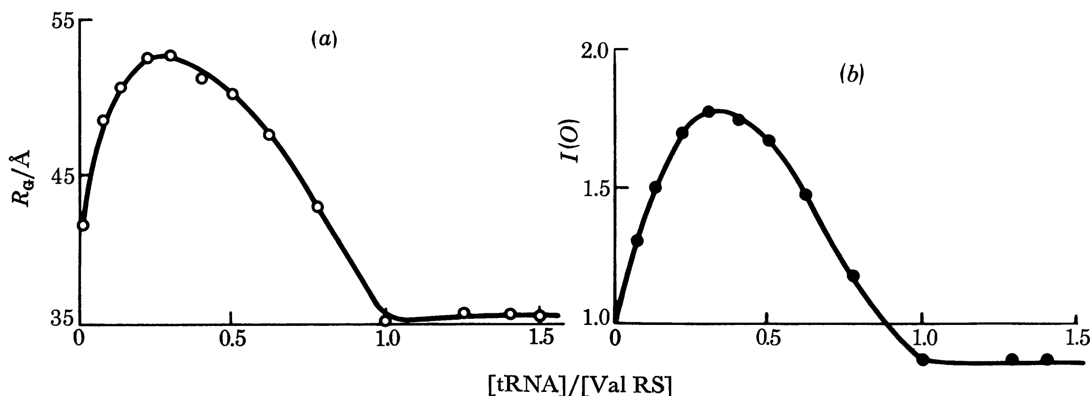

FIGURE 3. Titration of valyl tRNA synthetase from yeast with the corresponding tRNA: (*a*) the radius of gyration; (*b*) the intensity at the origin. Data are from buffer with 77 % D_2O, with 50 mM potassium phosphate, pH 6.3.

dissociated either by adding tRNA or by increasing the salt content of the solution. Their electrostatic origin is obvious, from the sensitivity to ionic strength. Conversely, the stoichiometric complex obtained in the presence of excess tRNA and which is made through specific interactions is not sensitive to ionic strength. One may speculate that in the cell the electrostatic interaction favours the contact between the two molecules and makes easier the recognition between cognate molecules.

(*c*) The change of conformation of the enzyme is illustrated in figure 3*a* for $tRNA_{Val}$ from yeast (from Zaccaï *et al.* 1979). The only structural information so far has been deduced from the radius of gyration. The data shown are for solution in a solvent in which the tRNA is

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nearly invisible. At small tRNA:enzyme ratio, the increase of R_G corresponds to the above discussed aggregation. But when the stoichiometric complex is formed (in that case a 1:1 complex) the radius of gyration decreases from 40 to 35 Å. So the formation of the specific complex is accompanied by a huge change of configuration of the enzyme. Figure 3*b* shows that this change of conformation is accompanied by a decrease in the intensity at the origin, which is given by (2). As the enzyme concentration is unchanged, and the tRNA does not contribute to the scattering, there are only two possible explanations. The first would be a change of Σb through a change of deuteration of exchangeable protons associated with the change of conformation of the enyzme. This is excluded by the reversibility of the phenomenon. So the only remaining explanation is a change of specific volume of the system. So we therefore believe that we have established that, in appropriate conditions, the interaction between tRNA and the cognate tRNA synthetases is accompanied by a decrease by 1 % of the specific volume. Our belief is that this is related to the modification of the hydration shells during interactions and the point is somewhat supported by the dependence of the phenomenon on ionic strength. This suggests that water may play a key role in protein–RNA interaction, and that the action of salt may be to some extent mediated through modification of the structure of the water in hydration shells.

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