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The triple isotopic substitution method in small angle neutron scattering. Application to the study of the ternary complex EF-Tu · GTP · aminoacyl-tRNA

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Abstract

The TIS (triple isotopic substitution) method in small angle neutron scattering was applied to determine the radius of gyration of polypeptide elongation factor Tu (EF-Tu) from *E. coli* associated with GDP and within the ternary complex EF-Tu · GTP · aminoacyl-tRNA. The results showed that, within errors of about 1 Å, there is no change in the radius of gyration of the EF-Tu moiety upon ternary complex formation. Experiments were performed in H_2O buffer, in which complex formation could be followed on an absolute scale because of the relatively large contrast of both protein and tRNA. The TIS method is based on the analysis of a scattering curve that is the difference between the scattering of two solutions containing appropriately deuterium labelled particles. A necessary condition for the application of the method is that the two solutions are identical in all respects except for the extent of deuterium label. The main properties of TIS that make it very useful for the study of complex particles in solution were confirmed by this study. These are the elimination of interparticle effects in the difference curve, the 'invisibility' of unlabelled parts of the particles and the independence of the difference scattering curve on the buffer ^2H_2O–H_2O content. The last property is of particular interest for the study of interactions that may be influenced by ^2H_2O, since, contrary to classical contrast variation methods, TIS experiments can be performed in H_2O buffer alone.

Keywords: Neutron scattering; Isotopic substitution; EF-Tu · GTP · aminoacyl-tRNA complex

1. Introduction

Neutron scattering was first applied to biological macromolecules in 1969, in experiments on hemoglobin solutions [1]. Since then, a varied methodology has been developed and widely used to solve problems in structural biology by small angle neutron scattering (SANS) from solutions. 'Contrast variation' is probably the best known of these approaches (review in Ref. [2]). The 'triangulation' method suggested by Engelman and Moore [3] and

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Hoppe [4] is more complicated. Developed primarily for measuring distances between labelled components of a particle, in solutions with high particle concentrations, it was applied to determine the distances between proteins in the 30S ribosome subunit of *Escherichia coli* [5]. A third method, developed to obtain the form factor of a particle in concentrated solutions, has been used extensively in the study of polymer solutions by SANS [6,7].

Three experimental schemes correspond to these methods, respectively. The first is based on subtracting the solvent scattering from that of the solution, which contains protonated or deuterated particles of the same type. Contrast variation or inverse contrast variation is based on the simple physical principle whereby a particle component in solution scatters neutrons only when its scattering density differs from that of the solvent. Contrast is proportional to this difference and it is varied by changing the scattering density of the solvent (different H_2O–D_2O (^2H_2O) mixtures) or of the particle (e.g. by extracting proteins from organisms grown in a deuterated medium). The second scheme is based on the subtraction of the scattering of a solution (II) from that of a solution (I) [3,4]. Solution (I) contains a mixture of particles of two types with deuterium-labelled pairs and unlabelled proteins. Solution (II) contains a mixture of particles of two other types in which one or the other of the protein pair is labelled by deuterium. The main advantage of this approach is that distances can be measured between the labelled pairs in macromolecular complexes by using high macromolecule concentrations. Its main disadvantage is that it can only be used to measure interlabel distances. In the third scheme, one also subtracts the scattering of the solvent from that of the solution, but in contrast to the first scheme, the solution contains a mixture of unlabelled and deuterated macromolecules. It is the method of double isotopic substitution. A single-particle scattering function can be calculated at any finite concentration of macromolecules in solution if such a measurement is repeated using another ratio of deuterated to unlabelled macromolecules in the mixture. The main limitation of the approach is that it can be used only with homogeneous particles such as polymers and is, therefore, not very suitable for biological samples.

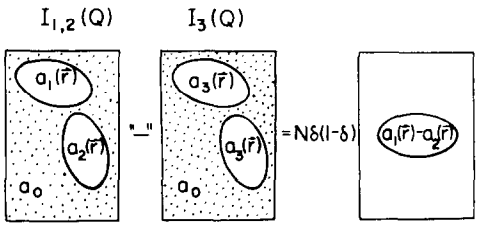

Fig. 1. General scheme of the TIS method. $I_{12}(Q)$ and $I_3(Q)$ are the scattering from solution (I) and (II); $a_1(r)$, $a_2(r)$ and $a_3(r)$ are the scattering densities of protonated, deuterated and semideuterated particles; N is the number of particles in solution, δ is the fraction of deuterated particles in solution (I).

We recently proposed the theoretical basis of a new approach for studying complex biological particles by SANS, the triple isotopic substitution (TIS) method [8,9]. The experimental scheme of TIS is described in Fig. 1. The scattering of solution (I) containing a mixture of protonated and deuterated particles and the scattering of solution (II) containing intermediately deuterated particles must be measured with identical concentrations. At a defined fraction of deuterated particles in the mixture, the difference scattering curve coincides with the scattering curve of the particle whose scattering density is equal to the difference of scattering densities for deuterated and unlabelled particles. The experimental scheme leads to interesting and novel predictions [8,9]. A protein or nucleic acid moiety of a nucleoprotein complex can be rendered invisible in ordinary light water (H_2O) solution, for example, contrary to the classical contrast variation schemes that require the use of ^2H_2O, which may have undesirable effects on biochemical interactions. The study of protein complexes with large ligands in light water offers many methodological advantages if complex formation can be controlled quantitatively.

The main goal of this paper is to briefly summarise the main theoretical and experimental background of the TIS method and to show that it can be successfully applied to the study of RNA-protein interactions by using, as an example, the EF-Tu-GDP and EF-Tu-GTP-aminoacyl-tRNA complexes of *E. coli*.

Polypeptide elongation factor Tu from *E. coli* belongs to the family of guanine-nucleotide binding proteins and it is one of the most extensively studied proteins. It participates in the polypeptide elongation

cycle as a carrier of elongator tRNA charged with amino acid (aminoacyl-tRNA) to the ribosome (review in Ref. [10]). During the elongation cycle, EF-Tu interacts with at least five ligands: GDP, GTP, aminoacyl-tRNA, elongation factor Ts and the ribosome. Atomic structures from X-ray crystallography have now been published for trypsin modified EF-Tu · GDP from *E. coli* [11] and EF-Tu complexed with non-hydrolysable GTP analogues from *Thermus thermophilus* [12] and *Thermus aquaticus* [13]. EF-Tu is made up of three structural domains, including a GDP binding domain that is very similar to that in other GDP binding proteins. From the crystallographic structures there appear to be major domain rearrangements between the GDP bound and the GTP analogue bound forms of EF-Tu [12,13].

2. Theoretical and experimental background

The theoretical principles underlying the method were derived by Pavlov and Serdyuk [8],

$$I_{1,2}(Q) - I_3(Q) = N\delta(1-\delta)I_F(Q), \quad (1)$$

$$a_3(\boldsymbol{r}) = (1-\delta)a_1(\boldsymbol{r}) + \delta a_2(\boldsymbol{r}), \quad (2)$$

where $I_{1,2}(Q)$ is the scattering of solution I containing the mixture of unlabelled and deuterated particles whose scattering densities are $a_1(\boldsymbol{r})$ and $a_2(\boldsymbol{r})$, respectively. $I_3(Q)$ is the scattering of solution (II) containing intermediately deuterated particles of scattering density $a_3(\boldsymbol{r})$, N is the number of particles in solution, δ is the fraction of deuterated particles in solution I, and $I_F(Q)$ is the scattering curve of a particle whose scattering density $a_F(\boldsymbol{r})$ is equal to the difference between $a_1(\boldsymbol{r})$ and $a_2(\boldsymbol{r})$,

$$a_F = a_1(\boldsymbol{r}) - a_2(\boldsymbol{r}). \quad (3)$$

For given scattering densities $a_1(\boldsymbol{r})$, $a_2(\boldsymbol{r})$ and $a_3(\boldsymbol{r})$ Eq. 2 is used to determine δ. For these equations to be valid, it is sufficient that the particle structure, interparticle interactions, and interaction of particles with the solvent do not depend on the extent of the particle deuteration.

The TIS method leads to the following predictions:

Prediction 1. The contribution of interparticle interference and particle association (e.g. dimerization) to the difference scattering curve is eliminated because in Eq. (1), $I_F(Q)$ pertains to the single particle.

Prediction 2. Because the exchange of labile hydrogen atoms between the particle and the solvent does not depend on particle deuteration, the difference scattering curve is independent of solvent isotopic content (D_2O fraction in the H_2O–D_2O mixture). This results from the main equations, especially Eq. (3). It also follows that distortions of the solvent near the particle surface do not influence the difference scattering curve (they cancel out in the subtraction: $a_1(\boldsymbol{r}) - a_2(\boldsymbol{r})$). This is important for studies of highly charged macromolecules such as tRNA, which are surrounded by a solvent layer that differs significantly from bulk solvent [14].

Prediction 3. Any 'small' or 'large' molecules present in equal concentrations in solutions (I) and (II) will be 'invisible' in the difference scattering curve. This prediction opens the way for the study of one of the components in a binary complex in the presence of an excess of the other.

Prediction 4. Any complex component of constant scattering density in the three types of particle will be invisible in the TIS analysis at any D_2O fraction in the H_2O–D_2O mixture. This also is a simple consequence of Eqs. (1) and (3), which state that the difference scattering curve pertains to a particle whose scattering density is the difference between the scattering densities of the particles of the first and the second types.

'Contrast' in the TIS method is the difference between the scattering densities of the labelled and unlabelled particles in solution I, i.e., $a_D(\boldsymbol{r}) - a_H(\boldsymbol{r})$. This is an important difference with other contrast variation techniques, where 'contrast' refers to the difference between the scattering densities of the particle and the solvent, i.e. $a_H(\boldsymbol{r}) - a_0$ (for an unlabelled particle in solvent of scattering density a_0). The difference between scattering densities of an unlabelled and completely deuterated protein is twice as large as the contrast of an unlabelled protein in H_2O. This results in approximately the same difference intensity in both methods in spite of the $\delta(1-\delta)$ term in Eq. (1).

The four predictions of the TIS method have been confirmed experimentally on EF-Tu from *E. coli* [15,16]. It was shown that particle association (some dimer formation in this case), the addition of 'small'

molecules (such as glycerol) or 'large' molecules such as pyruvate kinase) in solutions (I) and (II) in the same quantity had no influence on the difference scattering curve obtained. The possibility to extract single-particle scattering as well as normalised inter-particle interference functions from the TIS data was demonstrated [17]. This enabled the detection of the presence of aggregates in the solution, even in cases where the molar mass of a particle and/or its concentration were unknown.

3. Materials and methods

GTP, ATP, phosphoenolpyruvate (PEP) and pyruvate kinase (PK) were from Boehringer. [^{14}C]Leucine was from CEA, France and [^{3}H]GDP was from Amersham. Sepharose 4B and DEAE-Sepharose CL-6B were from Pharmacia. HEPPS was from Sigma and BAPP-sepharose was prepared as previously described [18]. All other chemicals were of analytical quality.

The following buffers were used in the scattering experiments. Buffer HMS: 94 mM HEPPS, 10 mM $MgCl_2$, 80 mM KCl, 0.5 mM DTT pH 7.5. Buffer HLS: 40 mM HEPPS, 5 mM $MgCl_2$, 0.5 mM DTT pH 7.5. All pH values were measured at room temperature.

EF-Tu-GDP with three levels of deuteration was isolated from *Escherichia coli* (MRE 600). To obtain completely deuterated EF-Tu, the growth medium contained 99.8% D_2O and D-succinate as carbon source. A medium containing 79% D_2O and H-glucose as carbon source was used to obtain intermediately deuterated protein. Fractionation of EF-Tu-GDP was carried out by a slightly modified procedure to that described previously [19]. All samples of EF-Tu-GDP in this study exhibited a single band on sodium dodecyl sulphate-polyacrylamide gel electrophoresis and had GDP binding activity of 21–22 nmol/mg.

Partially purified $tRNA^{leu}$ was isolated from total tRNA by successive chromatographic fractionation on BAPP-sepharose [18] and on sepharose 4B using a negative gradient of ammonium sulphate [20]. By the usual charging assay, the sample had a leucine acceptance value of 750 pmol/A_{260}, which corresponds to $\approx$ 50% pure tRNA. A sample of $tRNA^{phe}$ with a phenylalanine acceptance value of 1200 pmol/A_{260} was a generous gift from Dr. M. Ehrenberg (Uppsala). Before the scattering experiments $tRNA^{leu}$ was charged preparatively as described previously [17].

3.1. SANS measurements

SANS measurements were performed on the D11 small-angle camera at the Institut Laue–Langevin in Grenoble [21]. Samples were contained in quartz cells of 1.00 mm optical path length. Measurement times were between 15 and 60 min. The sample temperature was maintained at 6°C during the experimental runs: a neutron wavelength (λ) of 9.8 Å was used with an angular range corresponding to $0.012 < Q < 0.077$ Å^{-1} (the scattering vector amplitude $Q = (4\pi \sin \theta)/\lambda$, with θ, half the scattering angle).

3.2. Absolute measurements

The differential scattering cross-section per unit solid angle per unit volume of solution, $J(Q)$, was measured in absolute units (cm^{-1}) by using the incoherent scattering from water as a calibration [22]. For difference scattering (solution minus solvent or solution (I) minus solution (II)) it was more convenient to use the normalised value $I(Q) = J(Q)/(CN_A)$, where C is a particle concentration in g/cm^3 and N_A is Avogadro's number. The units for $I(Q)$ are barn/g (barn = 10^{-24} cm^2). The product $I(Q)$M is the scattering cross section (in barns) for one particle of molar mass M g/mol which can be compared with that calculated from its atomic composition. This comparison is a useful test for particle association in solution. When the number of particles (N) is omitted from Eq. (1) it becomes valid for normalised scattering cross-sections. Throughout the next sections, $I(Q)$ with corresponding subscripts denotes a normalised scattering cross section in barns/g.

3.3. Determination of the fraction δ of deuterated EF-Tu in the mixture

In a 'classical' contrast variation experiment, the 'match-point' of a particle is its mean scattering density expressed as a $D_2O:H_2O$ ratio. It is deter-

mined experimentally from the forward scattering in solvents with different $D_2O:H_2O$ ratios [2]. To determine the match points γ_H, γ_{HD} and γ_D for (H)-, (HD) and (D) EF-Tu, respectively, the SANS measurements were performed in different H_2O–D_2O mixtures in buffer HMS with protein concentrations of about 4 mg/ml. The match points obtained were 41%, 89% and 127% for the three particles, respectively. They are related to δ by

$$(1-\delta)\gamma_H + \delta_{\gamma HD} = \gamma_D. \quad (4)$$

This is equivalent to Eq. (2) in the case of a one component particle. The value of δ determined from Eq. (4) was 0.56.

4. Results

4.1. Conformational rearrangements of EF-Tu upon ternary complex formation with GTP and leu-tRNA^{leu} in H_2O

Two experiments were performed to study conformational rearrangements in EF-Tu upon ternary complex formation with GTP and aminoacyl tRNA (Fig. 2), one for EF-Tu-GDP and one for the protein within the complex. In the first, the TIS difference scattering curve for EF-Tu-GDP was measured by subtracting the scattering of solution (II) containing intermediately deuterated EF-Tu-GDP (HD-Tu) from that of solution (I) containing a mixture of unlabelled EF-Tu-GDP (H-Tu) and that of solution I containing a mixture of unlabelled (H-Tu) and deuterated (D-Tu) EF-Tu-GDP molecules. The optical densities (at 280 nm) of H-, D- and HD-Tu solutions were made equal by appropriate dilution of the concentrated stock solutions. EF-Tu-GDP concentrations were determined by using a value of 0.8 for A_{280} for 1 mg/ml of protein [23]. The H- and D-Tu solutions were mixed by volume with a simultaneous control by weight and SANS measurements were performed in the same cuvettes in which the UV measurements had been carried out. Fig. 3 (curve a) is the TIS difference scattering curve obtained.

All the scattering curves in Fig. 3 were plotted as ln $I(Q)$ versus Q^2. In the Guinier approximation that is valid at Q values close to $R_gQ \approx 1$ (see

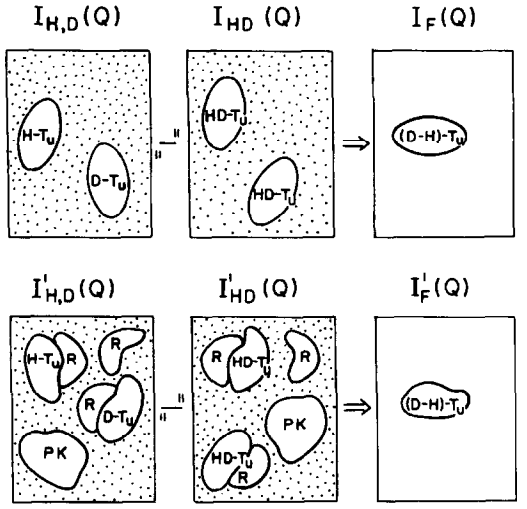

Fig. 2. General scheme of the experiments. (a) TIS experiments with EF-Tu-GDP, $I_{HD}(Q)$ is the scattering differential cross-sections of solution (I) containing the mixture of H-Tu and D-Tu, $I_{HD}(Q)$ is that of solution (II) of HD-Tu. (b) TIS experiment with the ternary complex EF-Tu·GTP·aminoacyl-tRNA, $I'_{HD}(Q)$ and $I'_{HD}(Q)$ are the scattering of solution (I) and (II), respectively, after addition of aminoacyl-tRNA (denoted as R on the figure) and pyruvate kinase (denoted as PK) to the mixture of H-Tu and D-Tu and to the solution of HD-Tu used in experiment (a). The difference in the shape of EF-Tu in (a) and (b) reflects possible conformational changes in EF-Tu upon complex formation.

below for the definition of R_g and Ref. [2], for example),

$$\ln I(Q) = \ln I(0) - \tfrac{1}{3}R_g^2Q^2,$$

and the zero angle scattered intensity, $I(0)$, and the square of the radius of gyration of contrast in the particle in solution, R_g^2, could be calculated from a straight line fit to the data in the small Q range.

In order to perform the second experiment, the components needed for ternary complex formation (leu-tRNA^{leu}, pyruvate-kinase, GTP, PEP) were added to both solutions (I) and (II) in equal amounts. The ternary complex EF-Tu-GTP-leu-tRNA^{leu} was prepared in a standard way; EF-Tu-GDP was converted into EF-Tu-GTP by incubation with 14 mM GTP, 14 mM PEP and 0.5 mg/ml PK for 30 min at 37°C. Then, the 50% pure leucyl-tRNA^{leu} was added in triple molar excess over EF-Tu to achieve saturation (EF-Tu and tRNA concentrations were 4.0 and 7.0 mg/ml, respectively). This experimental procedure is completely justified since the added 'small'

and 'large' molecules (like PK or non-bound tRNA molecules) do not contribute to the TIS difference scattering curve whereas the molecules of unlabelled leu-tRNA^{leu} and GTP participating in the ternary complex are 'invisible' as they can be considered as components of the constant scattering density in the complexes of the three types (which include H-, D- and HD-Tu). The experimental TIS difference curve of EF-Tu within the ternary complex is in Fig. 3 (curve b). The results of the SANS measurements are in Table 1.

The zero angle scattering cross sections (normalised by the concentration of EF-Tu) obtained in the two experiments (167.1 ± 4.3 and 169.5 ± 5.4 barn/g) coincide within experimental error (±5%), both values being very close to the TIS scattering cross section of the EF-Tu monomer (166.8 barn/g) calculated from its chemical composition, partial specific volume and the match points of H-Tu and D-Tu (see Ref. [16] for details). Moreover, the radius of gyration of EF-Tu-GDP obtained in the first experiment (23.6 ± 0.4 Å) coincides within the limits of error with the radius of gyration of EF-Tu in the ternary complex (23.9 ± 0.7 Å). This limits conformation changes in EF-Tu upon ternary complex formation to those that do not affect the radius of gyration. Note that the first three experimental points were excluded from the calculation of $I_F(0)$ and R_g as they were unreliable because too close to the edge of the beam-stop on the D11 detector.

Table 1
Experimental radii of gyration and scattering cross-sections at zero scattering angle in barn/g (per EF-Tu monomer) for complexes EF-Tu·GTP·Leu-tRNA^{Leu} with variously deuterated EF-Tu

	Complex with			
	H-Tu	HD-Tu	D-Tu	TIS difference
R_g (Å)	24.9 ± 0.2	24.0 ± 0.1	24.7 ± 0.1	23.9 ± 0.7
$I_{exp}(0)$	140.1 ± 1.4	321.2 ± 1.2	534.7 ± 2.0	169.5 ± 5.4

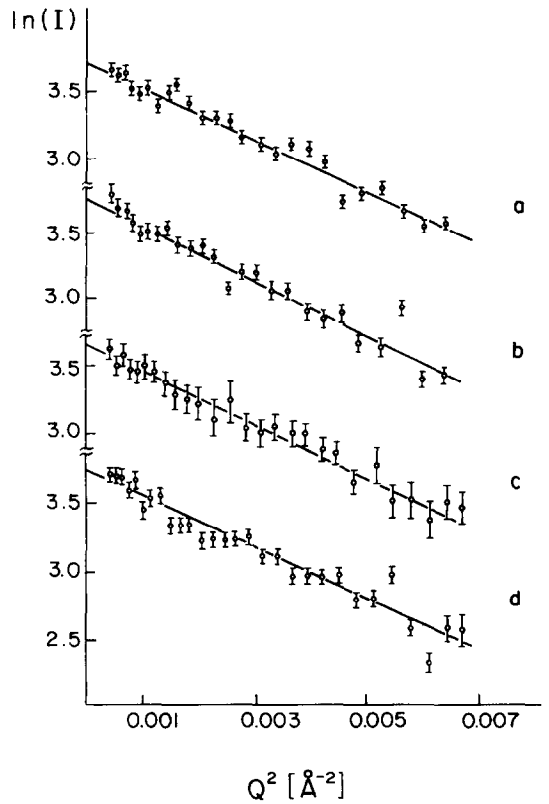

Fig. 3. Guinier plot of the TIS difference scattering curve for EF-Tu-GDP in H_2O in HMS buffer (a); compared with the Guinier plot of the TIS difference curve of EF-Tu in the ternary complex EF-Tu·GTP·Leu-tRNA^{Leu} in the same buffer (b). Guinier plot of the TIS difference scattering curve for EF-Tu-GDP in H_2O in HLS buffer (c); compared with the Guinier plot of the TIS difference curve of EF-Tu in the ternary complex EF-Tu·GTP·Phe-tRNA^{Phe} in the same buffer (d).

4.2. Conformational rearrangements in EF-Tu upon ternary complex formation with GTP and phe-tRNA^{phe} in H_2O

Curves c and d in Fig. 3 represent the TIS difference scattering curves for EF-Tu-GDP and the ternary complex EF-Tu-GTP-phe-tRNA^{phe}, respectively. These experiments, although similar to those described in the previous section, were performed in a slightly different manner. They were carried out using other preparations of HD- and D-EF-Tu (isolated from the same HD- and D-*E. coli* cells); another buffer (HLS) with low salt content was used for the SANS measurements; 75% pure tRNA^{phe} was used. Also, the ternary complex EF-Tu GTP-phe-tRNA^{phe} was prepared differently: EF-Tu-GDP (4.9 mg/ml) and tRNA^{phe} (5 mg/ml) were incubated together with 10 mM GTP, 10 mM PEP, 2

Table 2
Experimental radii of gyration and scattering cross sections at zero scattering angle in barn/g (per EF-Tu monomer) for complexes EF-Tu·GTP·Leu-tRNA^{Phe} with variously deuterated EF-Tu

	Complex with			
	H-Tu	HD-Tu	D-Tu	TIS difference
R_g (Å)	25.9 ± 0.2	24.2 ± 0.2	24.5 ± 0.2	23.4 ± 0.7
$I_{exp}(0)$	128.2.1 ± 1.5	302.9 ± 1.8	540.2 ± 1.5	170.4 ± 4.9

mM ATP, 0.2 mg/ml PK, 0.02 mg/ml MK and 80 units of phenylalanyl-tRNA synthetase for 15 min at 37°C to convert EF-Tu-GDP into EF-Tu-GTP and acylate tRNA^{phe}. The results of the SANS measurements are in Table 2.

Zero angle scattering cross sections (normalised by the concentration of EF-Tu) for free EF-Tu-GDP (166.0 ± 3.3 barn/g) and for EF-Tu in the complex with GTP and Phe-tRNAPh (170.4 ± 4.9 barn/g) obtained from the data in Fig. 3 (curves c and d) were the same within experimental error (±5%); the radius of gyration of EF-Tu-GDP obtained in the first experiment (23.6 ± 0.4 Å) was also identical with the radius of gyration of EF-Tu within the ternary complex (23.4 ± 0.7 Å).

The results obtained in this study are summarised in Table 3. They show clearly that for the two different sets of experimental conditions there is no measurable difference between the radii of gyration of EF-Tu-GDP and EF-Tu within the ternary complex with GTP and aminoacyl-tRNA.

Table 3
Summary of results obtained by the TIS method in this study

	Experiments in H_2O	
	complex with tRNA^{Leu}	complex with tRNA^{Phe}
$I_F(0)$ for EF-Tu	167.1 ± 4.3 barn/g	166.0 ± 3.3 barn/g
$I_F(0)$ for EF-Tu in the ternary complex	169.5 ± 5.4 barn/g	170.4 ± 4.9 barn/g
R_g of free EF-Tu	23.6 ± 0.4 Å	23.6 ± 0.4 Å
R_g of in the ternary complex	23.9 ± 0.7 Å	23.4 ± 0.7 Å

5. Discussion

The potential of the triple isotopic substitution method in the study of biological complexes by SANS was explored. With this method it was possible to avoid using D_2O buffers for the study of the complexes, which simplifies experimental procedures considerably and allows a direct monitoring of association events in solution. Since the component with constant scattering density in the three types of particle does not contribute to the TIS scattering curve, the method offers a unique possibility of making particle components 'invisible' for neutron scattering even in light water buffers.

This study also demonstrated unambiguously the absence of any significant change in the radius of gyration of the EF-Tu molecule upon ternary complex formation putting a strong constraint on possible models of the complex. The neutron scattering radius of gyration of trypsin modified *E. coli* EF-Tu-GDP calculated from its crystal coordinates [11] is 23.5 Å (Kernel, private communication) similar to the values found in the solution study. The same calculation has not been done for the GTP analogue bound forms because the coordinates are not yet available.

Recently, the radii of gyration of the 16S rRNA, total ribosomal protein and protein S4 have been measured in situ in the 30S ribosomal subunit from *E. coli* by the TIS method [24]. The data for the total protein and RNA were consistent with earlier findings, confirming the reliability of the method. Moreover, the value obtained for S4 is arguably one of the first high-quality estimates of the radius of gyration of a ribosomal protein in situ. This case set the limit as to the minimum size within an assembly that could be analysed by the TIS method. Protein S4 is about 3% of the total mass of the 30S subunits and a good signal-to-noise ratio was obtained with 10 mg/ml of material. Since considerable larger concentrations could be used (from prediction 1 in the theoretical section) it follows that the minimum part of a complex that can be analysed by TIS is about to 1–2%.

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