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Fluorimetric distance determination by resonance energy transfer

Ribosome-bound transfer RNA

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Abstract. Using the technique of singlet-singlet (Förster-type) resonance energy transfer, we have determined five distances in the programmed ribosome, either with the P site or with both the A and the P sites occupied. Two of the distances are new and two agree with earlier measurements; the fifth showed disagreement in detail with earlier results of others, but a consistent general trend. The distances substantiate a current model for the location of ribosomally bound tRNA, except in regard of the position of the 3′ end of P-site tRNA, which seems according to our results to lie too far away from the 3′ terminus of the 16 S RNA to be accomodated in the model. We present new evidence for the hypothesis that anomalously charged tRNA does not bind to the cognately programmed A site in the same way as does tRNA charged with an amino acid. Occupation of the A site restricts mobility of the 3′ end of tRNA in the P site.

Key words: Fluorescence, energy transfer, ribosome, tRNA

Introduction

The general location of the tRNA molecules bound in the A and P sites has long been known to be at the interface of the 30 S and 50 S subunits, on account of the assignment of the codon-anticodon site to the 30 S and the peptidyl transferase centre to the 50 S. However, information on a chemical level is still scarce: what there is comes from cross-linking studies, either with bifunctional reagents or, in one case, by direct photodimerisation of A-site tRNA with 16 S rRNA (reviewed by Ofengand et al. 1986). There is therefore still a need to complement these data by triangulation, and energy transfer between fluorescence labels bound to defined chemical groups of the ribosome offers a good way of providing such data.

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The development of a new method for the steady-state measurement of singlet-singlet resonance energy transfer (Epe et al 1983) and its application to give distance measurements that are consistent both with each other and with the crystallographically determined distance, where known (Epe et al. 1982, 1983; Steinhäuser et al. 1983), prompted us to investigate the ribosome-tRNA complex by this method.

Theoretical background

The transfer of electronic excitation energy from one chromophore to another by the singlet-singlet resonance mechanism (Förster 1951) is well known. It has frequently been used for the measurement of the distance between the two chromophores, as the probability of migration of energy is related inter alia to this distance. Usually, the chromophores are fluorescent, and the fraction of the population of the one kind of excited fluorophore (donor) whose excitation energy is transferred to the other kind (acceptor) is measured by monitoring the quenching of the donors' fluorescence and/or the enhancement of the acceptors'. This is commonly termed fluorescence energy transfer. Details of the theory can be found in text-books (Freifelder 1982; Cantor and Schimmel 1980) and in standard review articles (especially Stryer 1978). We have summarized the basic theory in the more general context of simultaneous transfer in both directions (Woolley et al. 1987).

The fraction of energy transferred from donor to acceptor depends upon *(i)* the overlap between the wavelengths emitted by the donor and those at which the acceptor can absorb, *(ii)* the medium separating the donor and the acceptor, represented here by its dielectric constant, *(iii)* their distance apart and *(iv)* the relative orientation of the two fluorophores. In principle, the distance is calculated by measuring the other four variables and the use of standard formulae;

in practice, there are problems associated with the relative orientation, which cannot be measured directly, and with the prohibitively high precision required of the quenching and enhancement measurements, since these effects often amount only to a few per cent.

Solutions for the orientation problem, based on fluorescence anisotropy, have been proposed by Dale and Eisinger (1975) and by Haas et al. (1978). The latter method is less rigorous but it is more easily applicable and is now in general use. It uses the emission anisotropy, and thus the mobility, of the two fluorophores to estimate the limits of error due to orientation.

The problem of precision is largely overcome by the separation method, which we have described earlier (Epe et al. 1983). With this method, in essence, the fluorescence signals of donor and acceptor on the macromolecule are measured in a steady-state fluorimeter and the two fluorophores are then separated from one another, for example by enzymic digestion, and the measurement is repeated. In this way, the small changes are observed on one and the same sample, and serious error introduced by comparing samples is avoided. The separation method is also particularly suitable for computer analysis of entire spectra, and not just of maxima, which makes a further contribution to accuracy. In this way, fluorescence intensity changes down to a few per cent can be measured with sufficient accuracy for distance determination.

Experimental

Materials

tRNA was purchased from Boehringer Mannheim and labelled by literature methods, adapted when necessary (Friedrich 1984; Friedrich and Woolley 1988; Friedrich et al. 1988). Labels and labelling reactions used for the tRNA were: for the 3′ end, fluorescein thiosemicarbazide, attached by oxidation of the 3′ end with periodate; for the base s^4U_8, iodoacetylfluorescein; for the X base at position 34, fluorescein isothiocyanate. 16 S rRNA was extracted by phenol extraction of 30 S ribosomal subunits and labelled by 3′ oxidation with periodate and treatment with the fluorescent label as hydrazide. In each case, excess dye was removed by exhaustive re-precipitation with ethanolic buffer. Fluorescent labels were purchased from Molecular Probes Inc. (Plano, Texas) except for coumarin hydrazide, which was synthesized by Dr. B. Epe (Epe et al. 1983). 16 S-labelled 30 S subunits were prepared by standard reconstitution methods (cf. Traub et al. 1971). If required, they were combined with 50 S subunits and the resulting 70 S couples were isolated by sucrose density gradient centrifugation in *Tris* HCl (50 m*M*), NH_4Cl (100 m*M*), Mg acetate (10 m*M*), pH 7.5. 70 S and 30 S particles were assayed by poly(Phe) synthesis (Pon and Gualerzi 1976).

All labelled tRNAs were fingerprinted by digestion with RNase A and T_1 (Sigma, St. Louis, USA) and thin-layer chromatography (Steinhäuser et al. 1982a) to identify the labelled position. They were assayed by charging with amino acid (Friedrich 1984, based on Traub et al. 1971). Buffers used contained 20 m*M* magnesium in order to permit non-enzymic occupation of the A site (Epe et al. 1987) and to prevent dissociation of the complexes during measurement. The buffer used for complex formation, gel filtration and measurement contained NH_4Cl, 50 m*M*; Mg acetate, 20 m*M* for occupation of the A site, otherwise 10 m*M*; dithioerythritol, 0.1 m*M*; *Tris* HCl, 20 m*M*; pH 7.5 (Lührmann 1980). Oligonucleotides were synthesized as described by Lührmann (1980).

Preparation of tRNA-ribosome complexes

P site: In the smallest possible volume (typically 250 µl), 100 pmol (3.8 A_{260}) 70 S ribosomes, 10–30 nmol (0.2–1 A_{260}) oligonucleotide messenger and 200 pmol (0.1 A_{260}) tRNA were incubated for 5 min at 37 °C. The mixture was then chilled in ice and the complexes were isolated by gel filtration at 4 °C on a Biogel A 0.5 column (50–100 or 100–200 mesh). The messenger used was: for $tRNA_f^{Met}$, AUG; for $tRNA^{Tyr}$, $UA(U)_4$; for $tRNA_2^{Glu}$, $G(A)_5$ and for $tRNA^{Phe}$ $(U)_6$. The product was put on ice and used as soon as possible for fluorescence measurement. When the time came for the measurement to be made, 250 µl of the solution of the complex was pipetted into an empty quartz cell and another 250 µl – in an identical manner – into a quartz cell containing RNases T1 (300 units) and A (3 µg) in 6 µl buffer plus 25 µl saturated KCl solution to prevent precipitation of ribosomal proteins (cf. Epe et al 1982).

A site: The complex [70 S.$tRNA_f^{Met}$.$AUG(U)_3$] was generated by incubation as above; however, the incubation at 37 °C was extended to 10 min, after which 200 pmol (0.1 A_{260}) labelled $tRNA^{Phe}$ was added. After a further 10 min at 37 °C, the mixture was chilled and gel-filtered as above. Finally, an additional aliquot of messenger oligonucleotide was added, in order to compensate for that removed in the gel filtration. If the ribosomes were left unprogrammed, by omission of messenger, then no complex was formed.

Fluorescence methods

The samples needed for a single distance measurement are: *(i)* complex labelled with both donor and acceptor, *(ii)* complex labelled with acceptor only *(iii)* complex labelled with donor only and *(iv)* unlabelled complex. The fourth serves as a blank for background correction.

The spectra are taken in the following order.

(i) Emission spectra are taken on all four undigested samples, with an excitation wavelength chosen so as to excite both the donor and the acceptor such that neither's emission swamps that of the other. The limits of emission wavelength scanned need not be very wide as long as they embrace the bulk of the donor and acceptor emission peaks.

(ii) An excitation spectrum of the acceptor-only sample and an emission spectrum of the donor-only sample are taken, for calculation of the overlap integral and quantum yield (see below); here, the limits of wavelength scanned should be wide in order to provide an accurate integral.

(iii) The spectra of the four digested samples are taken in a manner identical to those in *(i)* above.

(iv) Appropriate standard dye solutions of unbound donor and acceptor dyes measured in a manner similar to *(iii)*. The ratios of intensities between these spectra and the components of the spectrum of the doubly-labelled sample in *(iii)* are used to calculate the degrees of labelling in the doubly-labelled complex.

(v) Finally, the concentration of ribosomes in the doubly-labelled sample of *(i)* is measured by absorption at 260 nm (1 nmol ribosomes $\equiv$ 38 A_{260}).

The fluorescence intensity registered is proportional to the intensity emitted by the sample only if certain instrumental artefacts are corrected for. One important correction is the "anisotropy correction", made by using polarising filters in the excitation and emission paths. If the empirical fluorescence intensity with a vertically polarised input beam and a horizontally polarised output beam is F_{VH}, and so forth, then the total fluorescence intensity F is given by

$$F = F_{VV} + 2\,F_{VH}\,F_{HV}/F_{HH}$$

and the anisotropy by

$$A = (F_{VV} - F_{VH}\,F_{HV}/F_{HH})/F\,.$$

All spectra were corrected in this way, so that the intensities registered were independent of anisotropy. (The ratio F_{HV}/F_{HH} reflects the wavelength-dependent response of the emission monochromator. It is therefore the same for all samples and need only be measured once.) Another correction is the "quantum correction", used to make the fluorescence intensity proportional to the number of quanta emitted at every wavelength. This correction (details in Steinhäuser et al. 1982b) was not usually needed, and was only made if spectra were to be used for calculations of overlap integral or quantum yield.

The spectra were taken on an SLM 8000 DS scanning, photon-counting fluorimeter adapted to take a thermostatted 7 × 7 mm quartz cell. The excitation bandwidth was 4 nm and the emission bandwidth 4 or 8 nm. Light-scattering caused no interference except in long scans ((ii) above) where the emission range included the excitation wavelength. The sharp Rayleigh scattering peak was removed by interpolation; the contribution of this procedure to the error in the integration was negligible. Raman scattering was eliminated by background subtraction: for labelled complexes, the spectrum of a corresponding unlabelled complex was subtracted (cf. above); for free dyes, an appropriate buffer spectrum was subtracted. Measurements were made at 20 °C. The stability of the complexes was checked by their fluorescence emission anisotropy. In the experiments described here, the impossibility of keeping the complexes for longer than a few hours on ice and even less time at 20 °C made it imperative to organise with extreme efficiency the taking of spectra as described above. Earlier publications may be consulted for further details of the basic fluorescence measurements (Steinhäuser et al. 1982b), the practical measurement of energy transfer by the separation method (Epe et al. 1982) and its theoretical background (Epe et al. 1983).

Quantum yields were measured by comparison with fluorescein sodium salt at pH 13 ($Q = 0.92$; Odom et al. 1980). Degrees of labelling were measured by comparison with fluorescein isothiocyanate, fluorescein thiosemicarbazide and the 2-mercaptoethanol conjugate of iodoacetyl fluorescein in known concentrations at pH 7.5. Coumarin labels were assayed by UV absorption ($\varepsilon_{460} = 35{,}000\,l\,mol^{-1}\,cm^{-1}$).

Calculation of distances

Distances were calculated by the use of standard formulae and, for certain steps in the separation method, by formulae stated elsewhere (Epe et al. 1983). To orient the reader, we give a brief summary; for detailed explanation, see Steinhäuser et al. (1982b), Epe et al. (1982, 1983) and standard texts (Freifelder 1982; Cantor and Schimmel 1980; Kuhn 1977).

For each distance to be measured, the emission spectrum of the undigested doubly-labelled sample, after background subtraction, was decomposed numerically into its two components arising respectively from the quenched donor and the enhanced acceptor (cf. (i) above; numerical procedures in Epe et al. 1983). Representative cases are illustrated in Figs. 1 and 2, discussed below. The coefficients of these components are x_D and x_A respectively. The procedure was repeated for the digested doubly-labelled sample, for which the donor is now unquenched and the acceptor unenhanced, giving coefficients x'_D and x'_A. The ratio x_D/x'_D is a measure of the degree of energy transfer observed by donor quenching E_{DQ}. Analogously, x_A/x'_A leads to E_{AE}. The explicit formulae are

$$E_{DQ} = (1 - x_D/x'_D)/\chi_a$$

and

$$E_{AE} = (x_A/x'_A - 1)\,\varepsilon_A/\chi_d\,\varepsilon_D$$

where ε is the absorption coefficient and χ_d (χ_a) is the degree of labelling with donor (acceptor) in the doubly-labelled sample. χ's are defined as the average number of dye molecules per complex. This is normally less than 1.0; we have shown elsewhere that the higher value sometimes found, due to double labelling of the oxidised 3′ end of RNA, does not introduce substantial error (Epe et al. 1982).

According to Förster (1951), the distance R between the fluorophores is then given by

$$R_{DQ(AE)} = R_0\,(E^{-1}_{DQ(AE)} - 1)^{1/6}$$

where R_0 is the distance between the fluorophores when E is 0.5. Thus two separate experimental values of R are obtained. R_0 in Ångstrøm units is calculated by the standard formula

$$R_0^6 = 8.79 \times 10^{-5}\,\varkappa^2\,n^{-4}\,Q_D\,I_{DA}$$

where Q_D is the quantum yield of the donor, $\varkappa^2$ is the orientation factor ($\varkappa^2 = 2/3$), n is the refractive index ($n = 1.4$) and I_{DA} is the overlap integral defined by

$$I_{DA} = \int_0^\infty F_D(\lambda)\,\varepsilon_A(\lambda)\,\lambda^4\,\mathrm{d}\lambda \Big/ \int_0^\infty F_D(\lambda)\,\mathrm{d}\lambda$$

where λ is wavelength.

Results

The points used for fluorescence labelling with a view to their triangulation were: the 3′ end of the 16 S RNA; the 3′ ends of A-site-bound and P-site-bound tRNA; position 8 of tRNA in the P site; and position 34 of tRNA in the P site. Since the methods and formulae are stated elsewhere (Epe et al. 1982, 1983; Steinhäuser et al. 1982 b), we display the data and the results accruing therefrom in a single table (Table 1).

Some representative spectra are given in Figs. 1 and 2. Figure 1 shows the anisotropy-corrected spectra for 3′-coumarin-16 S as a donor and 8-fluorescein-tRNA$_f^{Met}$ (P site) as acceptor, with excitation wavelengths of 460 nm (left) and 480 nm (right). The adequacy of the curve fit (dotted and solid lines) is clear. Figure 2 shows two more examples. On the left (donor, 3′-coumarin-16 S; acceptor, 8-fluorescein-tRNA^{Phe}, P site) the fit is also good, in spite of the lower signal-to-noise ratio. On the right (donor, 3′-coumarin-tRNA$_f^{Met}$, P site; acceptor, 3′-fluorescein-16 S), the fit is less satisfactory and it appears that the limit of the method is being approached. Cases showing worse fit were not evaluated.

The anisotropy values of the labels in the 70 S complexes are given in Table 2. An interesting case is the

Table 1. Summary of fluorescence data for energy-transfer measurements

No.	Donor	Acceptor	λ_{ex} [nm]	x_D/x'_D	χ_a	E_{DQ}	x_A/x'_A	ε_A [l mol^{-1} cm^{-1}]	ε_D [l mol^{-1} cm^{-1}]	χ_d	E_{AE}	Q_D	I_{DA} [l mol^{-1} cm^{-1} nm^4]	R_0 [Å]	R_{DQ} [Å]	R_{AE} [Å]
1	[3′-Coum]-16 S	[3′-FTSC]-tRNA$_f^{Met}$(P)	460	0.956	0.49	0.0892	0.967	22,800	35,000	0.75	–	0.62	1.76×10^{15}	49.7	73	–
2	[3′-Coum]-16 S	[3′-FTSC]-tRNA$_f^{Met}$(P)	480	0.951	0.49	0.1001	0.975	40,900	19,800	0.75	–	0.62	1.76×10^{15}	49.7	72	–
3	[3′-Coum]-16 S	[8-IAF]-tRNA$_f^{Met}$(P)	460	0.908	0.54	0.1702	1.100	18,800	35,000	0.75	0.0719	0.62	1.69×10^{15}	49.3	64	76
4	[3′-Coum]-16 S	[8-IAF]-tRNA$_f^{Met}$(P)	480	0.940	0.54	0.1107	1.058	34,150	19,800	0.75	0.1338	0.62	1.69×10^{15}	49.3	70	67
5	[3′-Coum]-16 S	[34-FITC]-tRNA^{Tyr}(P)	460	0.916	0.24	0.3515	1.070	22,000	35,000	0.55	0.0795	0.37	1.82×10^{15}	50.0	55	75
6	[3′-Coum]-16 S	[34-FITC]-tRNA$_2^{Glu}$(P)	460	0.723	0.56	0.4951	1.116	22,000	35,000	0.55	0.1323	0.37	1.67×10^{15}	49.2	49	67
7	[3′-Coum]-tRNA$_f^{Met}$(P)	[3′-FTSC]16 S	460	0.751	0.76	0.3279	1.740	22,800	35,000	1.48	0.3256	0.69	1.84×10^{15}	50.0	56	56
8	[3′-Coum]-tRNA$_f^{Met}$(P)	[3′-FTSC]-tRNA^{Phe}(A)	460	0.957	0.069	0.6245	1.765	22,800	35,000	1.28	0.3891	0.69	1.73×10^{15}	49.5	45	53
9	[3′-Coum]-16 S	[8-IAF]-tRNA^{Phe}(P)	460	0.888	0.80	0.1404	0.989	18,800	35,000	0.55	–	0.62	1.71×10^{15}	49.4	69	–
10	[3′-Coum]-16 S	[3′-FTSC]tRNA^{Phe}(A)	460	0.966	0.04	0.8436	1.200	22,800	35,000	0.55	0.2369	0.62	1.65×10^{15}	49.1	37	60

For details of calculations, see sources in text. (P), P site; (A), A site; λ_{ex}, excitation wavelength; x_D/x'_D, x_A/x'_A, ratios of coefficients for curve-fitting (Epe et al. 1983); χ_d, χ_a, degrees of labelling of donor and acceptor (labels per ribosome); E_{DQ}, E_{AE}, fraction of donor energy transferred to acceptor as measured by donor quenching or acceptor enhancement, respectively; Q_D, quantum yield of donor, I_{DA}, overlap integral for donor emission and acceptor excitation; ε_A, ε_D, extinction coefficients of acceptor and donor; R_0, Förster distance, for 50% energy transfer; R_{DQ}, R_{AE}, distances calculated on the basis of donor quenching and acceptor enhancement, respectively

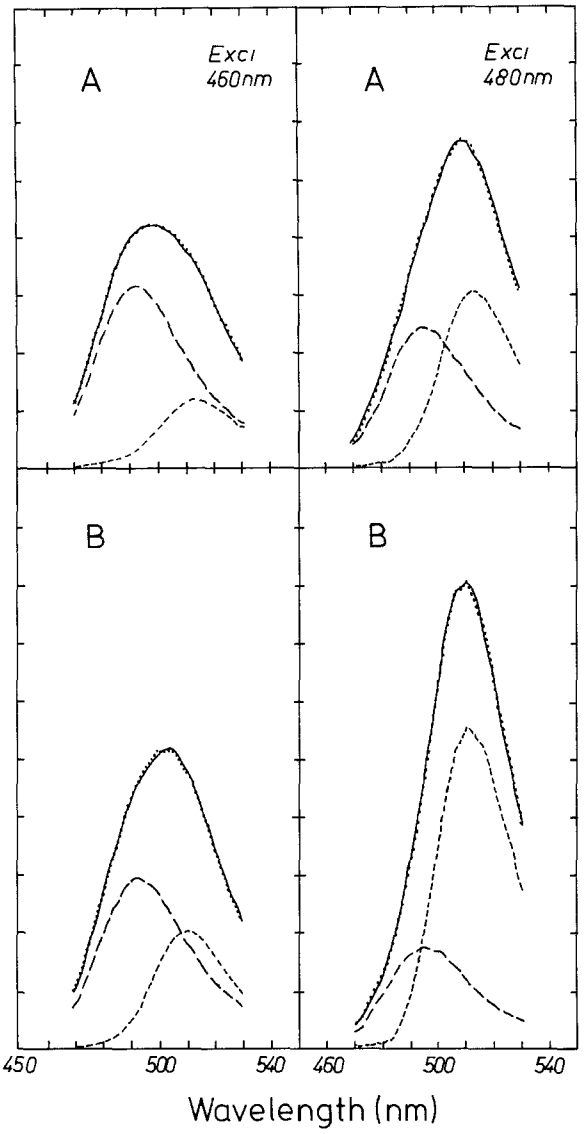

Fig. 1. Representative anisotropy-corrected spectra used to determine degree of energy transfer by the separation method. Donor, coumarin at 3′ end of 16 S RNA; acceptor, fluorescein at position 8 of $tRNA_f^{Met}$ (P site). *Left:* Excitation wavelength 460 nm; *right:* 480 nm. **A** Before separation of fluorophores (digestion with RNases); **B** after separation. *Full line:* fluorescence emission from sample labelled with donor and acceptor; *long-dashed line:* labelled with donor only; *short-dashed line:* labelled with acceptor only; *dotted line:* linear combination of donor-only and acceptor-only curves giving best fit to double-label curve

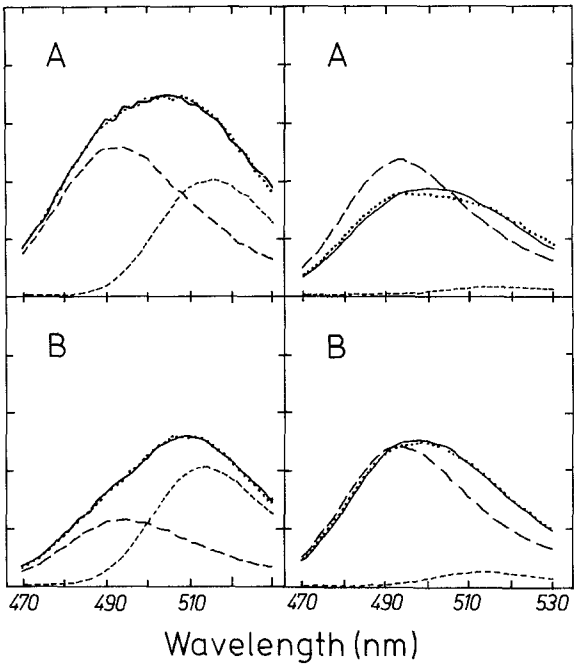

Fig. 2. Further spectra used to determine degree of energy transfer. Excitation wavelength, 460 nm. *Left:* Donor, coumarin at 3′ end of 16 S RNA; acceptor, fluorescein at position 8 of $tRNA_f^{Met}$ (P site). *Right:* Donor, coumarin at 3′ end of $tRNA_f^{Met}$ (P site); acceptor, fluorescein at 3′ end of 16 S RNA. For further explanation, see caption to Fig. 1. The quality of the curve-fitting is clearly not so good in the case on the right as in the other cases (see text)

Table 2. Anisotropy values of labels in 70 S complexes

Label	Fluorescent molecule in 70 S	Anisotropy
Coumarin	[3′-Coum]-16 S	0.35
Coumarin	[3′-Coum]-$tRNA_f^{Met}$ (P)	0.35
Fluorescein	[3′-FTSC]-$tRNA_f^{Met}$ (P)	0.23
Fluorescein	[3′-FTSC]-$tRNA_f^{Met}$ (P)*	0.32
Fluorescein	[8-IAFl]-$tRNA_f^{Met}$ (P)	0.27
Fluorescein	[8-IAFl]-$tRNA^{Phe}$ (P)	0.22
Fluorescein	[34-FITC]-$tRNA^{Tyr}$ (P)	0.20
Fluorescein	[34-FITC]-$tRNA_2Glu$ (P)	0.20
Fluorescein	[3′-FTSC]-$tRNA^{Phe}$ (A)	0.10

(P), in P site; (A), in A site; * A site occupied

anisotropy of 3′-end-labelled $tRNA_f^{Met}$ in the P site. Its value rises from 0.23 to 0.32 when the A site becomes occupied. We also note that the anisotropy of the label in P-site-bound [8-Fl]-$tRNA_f^{Met}$ is higher than that of the label in P-site-bound [8-Fl]-$tRNA^{Phe}$; the apparently tighter binding of the initiator tRNA to the P site is consistent with its function in protein synthesis.

The accuracy of the distance determination has been discussed exhaustively elsewhere (Odom et al. 1980; Epe et al. 1982, 1983; Steinhäuser et al. 1983; Friedrich 1984). We summarise here by stating that the greatest uncertainty is due to the orientation factor and leads to an expected mean error in R_0 of $\pm 15\%$, based on the anisotropy values of the labels (Haas et al. 1978). This figure is higher than usual, owing to the relatively high anisotropy (immobility) of the coumarin labels bound in 70 S complexes. For the rather large distances involved here, the correction for

"reverse" energy transfer from acceptor to donor (Woolley et al. 1987) can be ignored.

Stable occupation of the A site proved difficult to achieve. Various complexes were prepared that dissociated on the gel-filtration column. The only one showing sufficient stability was the [70 S.AUGUUU. $tRNA_f^{Met}$.$tRNA^{Phe}$] complex described here. This is consistent with the observations of others (e.g. Holschuh et al. 1981) that the A site has a lower binding constant for tRNA than the P site has.

For every donor-acceptor pair, meaningful x-coefficients ($x_D \leqq x'_D$, $x_A \geqq x'_A$, Table 1) were obtained. Only in experiments 1, 2 and 9 were x_A and x'_A very close, with $x_A < x'_A$, so that the acceptor enhancement could not be used to calculate the distance. The donor quenching in these three experiments gave distances close to the upper limit of the measurable range (see Discussion).

The energy transfer method is self-checking, by virtue of the double determination (R_{DQ} and R_{AE}) and, for the digestion method, of the overdetermined parameters obtained from curve fitting. The latter makes possible the evaluation of x_D/x'_D and x_A/x'_A with high accuracy. We can thus be confident that the distances found lie within the $\pm 15\%$ scatter expected on the basis of the orientation factor (see above). The average of all the groups lay within this range, except for the group in experiments 5 and 6, with a scatter of 19%, which is acceptable, and the distance from 16 S RNA (3′) to A-site-bound tRNA (3′) (experiment 10). In the latter case, the reason is clear from Table 1: χ_a is very low, so that the donor quenching observed is small and R_{DQ} subject to large error. We thus accept R_{AE} and reject R_{DQ} in this case.

Discussion

The five distances reported here are summarised in Table 1. Three of them can be compared directly with measurements by other groups. Distance measurements between further specifically-labelled points on 70 S ribosomes occupied in P and A sites (e.g. Fairclough and Cantor 1977; Johnson et al. 1982; Paulsen et al. 1983) are neither supported nor contradicted by our results, since they span positions of tRNA that are geometrically distant from those covered here.

(i) The 16 S RNA (3′ end) to P-site tRNA (3′ end) was found by Robbins et al. (1981) to be 67–74 Å, in agreement with our value.

(ii) These authors also found the distance from 16 S RNA (3′ end) to P-site tRNA (base 37) to be greater than 61 Å. We find a distance of 62 ± 12 Å from 16 S RNA (3′ end) to P-site tRNA (base 34). In most representations of tRNA, bases 34 to 37 are stacked, so the difference between their positions should not be great in this context – especially as the tRNA in the P site is probably "seen" from the side by the 3′ end of the 16 S RNA (see below).

(iii) The distance between the 3′ ends of A-site and P-site tRNA was found earlier (Wells and Cantor 1980) to be 34 Å, while we find 49 Å. This discrepancy may be explained by experimental error in the fluorescence method, in which we prefer to adopt the value given by the separation method, for reasons stated. The alternative explanation, that some tRNA has dissociated from the A site in the cell, we discount because *(i)* the anisotropy of A-site-bound label, although low, was accurately reproducible, *(ii)* complex that survived the gel filtration step was then stabilised by added messenger, as described above, and *(iii)* there was no indication of dissociation during measurement, which should have been seen by changes in spectra with time and by poor curve-fitting.

In fact, the question of interest is not whether the 3′–3′ separation of the tRNAs is 34 Å or 49 Å but why it is so large, when these molecules should be able to react with one another at the peptidyl transferase centre. Possible reasons are: *(i)* The ribosome may check the size of incoming tRNA, as part of the mechanism for avoiding errors in translation; such a steric "sieve" is easily imaginable. Thus the 3′ end of tRNA bearing a bulky label would lie in a false position. *(ii)* The labelled tRNA is recognised as uncharged and binds in a different position, perhaps that associated with the stringent response (Wells and Cantor 1980). We believe that the latter is unlikely, unless the ribosome has some mechanism that identifies positively the amino acid at the 3′ end of incoming tRNA and rejects other groups bound to the ring at the 3′ end. We therefore find the first reason more plausible. Thus, tRNA charged with an anomalously bulky group does not bind to the cognately programmed A site in the same way as does tRNA charged with an amino acid. The low anisotropy of the 3′ label of A-site-bound tRNA confirms the high degree of segmental mobility at the 3′ end associated with this incorrect mode of binding.

Equally interesting is the rather large decrease in segmental mobility of the 3′ end of P-site-bound tRNA when the A site becomes occupied. It is frequently asserted that the A site first comes into being when the P site is occupied. The observed anisotropy change shows a structural feedback effect from tRNA in the A site to tRNA in the P site which confirms the physical intimacy of their relationship.

Hardesty and co-workers (1986) have observed a difference between the positions of uncharged tRNA (as used here) and acylaminoacylated tRNA (a better approximation to the natural peptidyl tRNA) in the P site. The largest difference seen was in the distance from protein L11 labelled at *Cys*-38 to P-site tRNA

labelled at position 47: this distance was (73 ± 9) Å for $tRNA^{Phe}$ and was >88 Å (error unstated) for Ac-Phe-$tRNA^{Phe}$. The structural and statistical significance of these results remain to be assessed (and the authors do not state what method was used to measure energy transfer, or whether the 70 S complexes were separated from unbound tRNA). However, there is no conflict between movement of the approximate magnitude indicated by Hardesty et al. (1986) and the trigonometrical data provided in the present work.

To compare our distance data with a current ribosome model, we refer to that of Ofengand et al. (1986), in which the anticodon region for the A site is in the cleft between the 30 S head and large lobe and that for the P site is further round towards the 30 S–50 S interface (cf. especially the 70 S view in Fig. 27.7 of Ofengard et al. (1986)). Reference to this figure indeed supports a distance of the order of 60 Å from the 3′ end of 16 S RNA (on the large lobe) to each of the labelled positions on P-site tRNA. Only the distance to the 3′ end of the tRNA appears significantly longer on the model than in our measurements (even though these are somewhat biased toward the short side by experiment 7 – see above). It remains to be seen whether the tRNA position on the model will be refined. However, we note a degree of uncertainty in the positioning of the 3′ ends in the model, since it places them rather far from the presumed peptidyl transferase centre.

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