Supporting Text

Preparation of *Escherichia coli* Ribosomes

Expression and Purification of *E. coli* Ribosomes. Crude ribosomal particles were prepared at 4°C from *E. coli* MRE600 cells grown in minimal medium containing $^{15}NH_4Cl$ (Campro Scientific, Berlin) as the sole nitrogen source, according to a modified version of the method of Bommer *et al.* (1). Eighty-seven grams of cells was washed in 250 ml of Tico buffer (20 mM Hepes•KOH, pH 7.6/6 mM magnesium acetate/30 mM ammonium acetate/4 mM 2-mercapthoethanol) and centrifuged at 16,000 × *g* [average centrifugal force (acf)] for 30 min at 4°C. The final cell pellet was resuspended in Tico buffer (3 ml per gram of cell pellet) and lysed by a French press (8,400 psi). One hundred microliters of a 0.1 M solution of DTT was then added to each 10-ml volume of lysate, and the solution was centrifuged twice (30,000 × *g* for 30 min, 4°C). The supernatant was then centrifuged at 50,000 × *g* (17 h, 4°C) and the resulting crude ribosomal pellet resuspended in Tico buffer to give 400-800 A_{260}. The pellets were then flash frozen in liquid nitrogen and stored in aliquots of 2,000 A_{260} at –80°C until further use. The preparation yielded ≈30,000 A_{260} of crude ribosomes, corresponding to 345 A_{260} of crude ribosome per gram of wet cell pellet. Aliquots (containing 2,000-4,000 A_{260} units) of crude ribosomes were further purified through linear density gradients from 7.5-40% sucrose in a Beckman (Beckman Coulter) zonal Ti-15 rotor (18,000 rpm, 16 h, 4°C). The gradients were prepared in Tico buffer for the purification of 70S ribosomes and in dissociation buffer (20 mM Hepes•KOH, pH 7.6/1 mM magnesium acetate/200 mM ammonium acetate/4 mM 2-mercapthoethanol) for preparation of the 30S and 50S ribosomal subunits. The fractionated ribosomal particles obtained from the gradient were concentrated by centrifugation at 50,000 × *g* (24 h, 4°C) for 70S ribosomes and at 10,0000 × *g* (20 h, 4°C) for 30S and 50S ribosomal subunits. The resulting ribosomal pellets were resuspended in 10 mM potassium phosphate buffer, pH 7.0/10 mM magnesium acetate/100 mM potassium chloride, to give a final concentration of 600-2,000 A_{260}.

Concentration Measurements. The concentration of ribosomes in the various solutions was determined using A_{260} measurements, where 1 A_{260} corresponds to 24 pmol/ml of 70S ribosomes, 36 pmol/ml of 50S subunits, and 72 pmol/ml of 30S subunits. The concentration of ^{15}N labeled titin used for quantitative analysis of the NMR peak intensities arising from the 50S ribosome was determined by using amino acid analysis (Protein and Nucleic Acid Facility, University of Cambridge).

Quality Controls of Ribosomal Particles. The homogeneity and integrity of the ribosomal preparations were estimated from analytical sucrose density gradients (10-30%) prepared in the same buffer as that in which the particles were stored and performed in a SW40 rotor at 18,000 rpm for 18 h at 4°C. Ribosomal RNA integrity was confirmed by electrophoresis on a 3.1% acrylamide/bisacrylamide (37.5:1) TBE buffer (90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3/urea gel). The protein content was analyzed by 2D gel electrophoresis according to Funatsu *et al.* (2) and by 1D SDS/PAGE. Poly(U)-dependent poly(Phe) synthesis was performed as described by Bommer *et al.* (1) for three independent sets of NMR experiments and on four different samples: (A) control ribosome; (B) ribosomes subsequent to >10 h of NMR acquisition; (C) resuspended ribosomal pellet after centrifugation of sample A; and (D) a resuspended ribosomal pellet after centrifugation of the ribosomes from B. Typical results obtained from these assays showed the following incorporation (pmol) of Phe per ribosome: (A) 208, (B) 278, (C) 186, and (D) 222, i.e., the control ribosomes (A and C) are highly active with the pelleted sample (C), having 89% of its initial activity value (A). The ribosomes used for NMR experiments (B and D) also indicated high activity, with the pelleted ribosomes (D) showing 80% of their initial activity in B.

Elongation factor G (EF-G) was produced and purified according to a procedure detailed previously (3).

NMR Experiments

Pulsed-Field Gradient (PFG)-Diffusion Measurements. PFG diffusion experiments (4) were performed by using the heteronuclear multiple quantum correlation (HMQC)-type pulse sequence described by Dingley *et al.* (5) modified by removing the final two gradient pulses. This modification preserves the signals from both zero- and double-quantum coherences and generates a 2-fold gain in signal intensity, an essential factor for the experiments described here given the low concentrations of the samples. Although zero- and double-quantum coherences decay at different rates in the diffusion experiment, adding both coherences affects the estimated diffusion constants by <10%.

The signal intensity is attenuated in the presence of a gradient pulse and, if other factors are constant, the signal intensity (I) relative to that in the absence of gradients (I_0) is given by:

$$I/I_0 \approx \exp-[(\gamma_H)^2\delta^2(\Delta-\delta/3))D_TG^2]. \qquad [\mathbf{1}]$$

G, δ, and Δ are, respectively, the size of, the duration of, and the time between the PFGs, whereas γ_H is the ^{1}H gyromagnetic ratio, and D_T is the translational diffusion coefficient.

The diffusion coefficients of two proteins can be related to their macromolecular radii via the Stokes--Einstein equation, $D_T = k_bT/6\pi\eta_w r_H$, (where k_b, η_w, and r_H are Boltzmann's constant, the viscosity, and the hydrodynamic radius, respectively). Assuming constant densities and spherical particles (6), then:

$$M_1/M_2 = (D_2/D_1)^3, \qquad [\mathbf{2}]$$

(where M_1 and M_2 are the molecular masses, and D_1 and D_2 the diffusion coefficients of proteins 1 and 2, respectively). This approach has the advantage of avoiding the need to know the values of viscosity and the absolute values of the diffusion coefficients.

Fast Heteronuclear Single-Quantum Correlation (HSQC) Experiments. There are four copies of L7/L12 in the ribosomal particles. To determine how many of these

proteins have sufficient motional freedom to be visible in the HSQC spectrum, the crosspeak volumes of L7/L12 resonances were compared with those of the added monomeric protein, ^{15}N labeled titin-I27. Fast HSQC experiments were recorded because they yield H,N crosspeaks that can be integrated readily, as water flip-back is achieved without selective pulses; in this way, nonuniform signal losses that result from saturation transfer from water to amide hydrogens are reduced. Also, the fast HSQC experiment uses a relatively small number of pulses, which, together with the use of composite ^{15}N pulses (90_x-240_y-90_x) (7) during the H,N coherence transfer steps, reduce intensity variations due to ^{15}N offset effects.

To confirm that the integrals were comparable, a number of precautions were taken: (*i*) A 3-s relaxation delay was used to ensure complete R_1 relaxation; (*ii*) crosspeak integration (rather than simple height measurement) was carried out, because R_2 relaxation of ^{1}H and ^{15}N nuclei causes linewidths to vary in each dimension of a 2D HSQC; and (*iii*) the delay in the INEPT experiment was shortened to 2.0 ms (the usual setting is 2.7 ms) to reduce the effects of systematic deviations of peak intensity resulting from amide hydrogen R_2 relaxation.

The possible effects of the R_2 relaxation times of amide nitrogens and protons on the signal intensities can be considered further: R_2 relaxation during t_1 and t_2 contributes only to the linewidths and cancels out through integration. The effect of apodization on integral ratios can empirically be shown to have a negligible effect. The loss of magnetization due to amide proton R_2 relaxation during the four INEPT delays Δ may, however, influence the observed crosspeak integrals and therefore the concentration comparison between L7/L12 and titin. To estimate this loss, we assumed an amide proton linewidth of 10 Hz for L7/L12, then $T_2 = 1/R_2 = 1/(\pi*\text{linewidth}) = 30$ ms [in line with those determined for the relatively fast relaxing globular C-terminal domain (CTD) of L7/L12]. The L7/L12 integrals are attenuated during the four INEPT delays Δ, by $1-\exp(-4*\Delta/30\text{ms})$, which for $\Delta = 2.7$ ms or 2 ms is equal to 0.3 or 0.23. These 23–30% losses are an upper limit (assuming no losses occur for titin) of the error on the concentration

comparison between L7/L12 and titin. We chose $\Delta = 2$ ms, i.e., crosspeak integrals of L7/L12 could be underestimated by, at most, 23% relative to those of titin crosspeaks.

^{15}N Relaxation Experiments. Experiments to determine the relaxation rates, R_1 (8) and R_2 [extracted from $R_{1\rho}$ (9, 10)], were performed at a ^{1}H frequency of 600 MHz (a ^{15}N frequency of 60.8 MHz). The recovery delay between transients was 1.5 and 2.0 s in the R_1 and R_1U experiments, respectively. R_1 relaxation experiments were recorded in an interleaved manner with randomly distributed relaxation delays of 10*, 100, 300, 500*, 600, 700, 800, 1,000, and 1,200 ms (* duplicate time points). Relaxation delays of 5**, 20*, 30*, 35, 40*, 45, and 60 ms (** triplicate time points) were used in $R_{1\rho}$ experiments. The spin-lock field strength used in the $R_{1\rho}$ experiment was $\omega_l = 1{,}587$ Hz. To avoid differential heating of the samples, a compensating ^{15}N pulse was applied during the recovery delay to ensure that the total energy absorbed by the sample was the same for all relaxation delays.

Integration of crosspeaks, curve fitting, and extraction of relaxation rates was carried out with the rate analysis package within NMRVIEW (11). The off-resonance rotating-frame relaxation rate $R_{1\rho}$ is given by (12):

$$R_{1\rho} = R_1 \cos^2\theta + R_2 \sin^2\theta + R_{ex} \sin^2\theta, \qquad [3]$$

where R_1 and R_2 are the longitudinal and exchange-free transverse relaxation rate constants, R_{ex} is the conformational exchange contribution to the transverse relaxation rate, $\theta = \arctan(\omega_1/\Delta\omega)$ is the tilt angle between the reduced static magnetic field $\Delta\omega = \omega - \omega_0$ and the effective field $\omega_e = (\Delta\omega^2 + \omega_1^2)^{1/2}$ in the rotating frame, ω is the spin-lock frequency, ω_0 is the ^{15}N Larmor frequency, and ω_1 is the spin-lock field strength. R_1 and $R_{1\rho}$ rates were determined by fitting the peak volumes to a two-parameter single-exponential decay function, and the errors in relaxation rates were estimated through Monte Carlo simulations of the relaxation data.

The τ'_m value for a given N-H^N vector in a molecule with a small degree of anisotropy depends on the angle ϕ between the relative orientation of the N-H^N vector and the principal axis of the diffusion tensor (13):

$$\tau'_m = \frac{\tau_l}{1 + \frac{\Delta}{2}\sin^2\phi} \qquad [4]$$

where $\tau_l = \tau'_{m(max)}$ and $\tau_s = \tau'_{m(min)}$ are, respectively, the longest and shortest τ'_m values and Δ ($= \tau_l/\tau_s - 1$), the difference between the value of the rotational anisotropy and unity.

Grid Search of Possible Orientations of the L7/L12 Domain. To examine whether the orientations of the helices derived in this way define a unique preferred orientation of L7/L12 relative to the ribosome, a coordinate system was set up with its origin at the amide nitrogen atom of Thr-52, the first residue in the crystallographic structure of the isolated CTD of L7/L12 (14). The *y*-axis was defined as the average orientation of the N-H^N bonds of the five residues used to determine $\tau'_{m(max)}$. Thus, according to Eq. **4**, the *y*-axis, defined above, should be parallel to the long axis of the diffusion tensor. The *xz* plane was then arbitrarily defined perpendicular to the *y*-axis, and the L7/L12 CTD was rotated around the *x*, *y*, and *z* axes in turn by using X-PLOR (15). This amounts to rotating the diffusion tensor relative the molecular structure to find domain orientation(s) consistent with the helical angles determined experimentally. A database of Euler angles defining each orientation and the angles of the α-helices relative to the *y*-axis was created, which was then analyzed systematically. A single unique value of the orientation tensor was found, after discarding those that are symmetry related. The helical angle orientations (Fig. 4*a*) are 66° (Helix I), 63° (Helix II), and 32° (Helix III) relative to the *y*-axis. These values are within the error margins of the experimentally determined helical angles given above and hence are consistent with the average orientation of the CTD of L7/L12 bound to the ribosome.

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