Binding of S21 to the 50S Subunit and the Effect of the 50S Subunit on Nonradiative Energy Transfer between the 3′ End of 16S RNA and S21†

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ABSTRACT: *Escherichia coli* ribosomal protein S21 was labeled at its single cysteine group with a fluorescent probe. Labeled S21 showed full activity in supporting MS2 RNA-dependent binding of formylmethionyl-tRNA$_f$ to 30S ribosomal subunits. Fluorescence anisotropy measurements and direct analysis on glycerol gradients demonstrate conclusively that labeled S21 binds to 50S ribosomal subunits as well as to 30S and 70S particles. The relative binding affinities are in the order 70S > 30S > 50S. Other results presented appear to indicate that S21 is bound in the same position on either 50S subunits or 30S subunits as in 70S ribosomes, suggesting that the protein is bound simultaneously to both subunits in the latter. Addition of 50S subunits to 30S particles containing probes on S21 and at the 3′ end of 16S RNA caused a decrease in the energy transfer between these points. The results correspond to an apparent change in distance from 51 to 61 Å.

S21 with a molecular weight of 8369 (Vandekerckhove et al., 1975) is the smallest and most basic protein of the *Escherichia coli* 30S ribosomal subunit. The sequence of its 70 amino acids is known (Vandekerckhove et al., 1975). It has a single cysteine residue at position 22 which can be labeled with sulfhydryl-reactive reagents under nondenaturing conditions. The protein has been mapped by immunoelectron microscopy near the constriction that separates the "head" from the "body" of the subunit (Stöffler et al., 1979; Lake, 1978).

Poly(4-thiouridylic acid) [poly(S^{4}U)][1] bound to 70S ribosomes was shown to react with S21 upon photoactivation as well as with S1 and S18 (Fiser et al., 1975). Cross-linking studies (Czernilofsky et al., 1975; van Duin et al., 1975; Heimark et al., 1976) place S21 near the 3′ end of 16S RNA, along with S1 and IF-3. A report (Poldermans et al., 1979) that S21 inhibits methylation of the adenines located 24 and 25 nucleotides from the 3′ end of 16S RNA in 30S subunits from kasugamycin-resistant strains of *E. coli* is further evidence for its location in this region.

Functional studies are also consistent with S21 being located near the 3′ end of 16S RNA. As originally proposed by Shine & Dalgarno (1974) and confirmed by Steitz & Jakes (1975), a sequence of 3–7 nucleotides located about 10 nucleotides 5′ to the initiator codon of natural mRNA appears to bind to a complementary sequence of contiguous bases at the 3′ end of 16S RNA. It has been demonstrated that S21 is necessary to bind Qβ RNA (Chang & Craven, 1977) and MS2 RNA (van Duin & Wijnands, 1981) to the 30S subunit, as well as for the binding of an octadeoxynucleotide complementary to the 3′ end of 16S RNA (Backendorf et al., 1981). From these studies, it has been proposed (van Duin & Wijnands, 1981) that the primary role of S21 is to help hold the 3′ end of 16S RNA in such a conformation that it can interact with natural mRNA.

There have been conflicting reports about the role of S21 in translating synthetic mRNAs such as poly(U). Held et al. (1974) found that addition of S21 to 30S ribosomes reconstituted in its absence caused a large increase in the ability to translate poly(U), while van Duin & Wijnands (1981) found that added S21 showed little effect on poly(U) translation but was required for reactions involving natural mRNA.

Here we report results of studies using S21 labeled at its sulfhydryl group with a fluorescent maleimide coumarin probe. The results indicate that S21 interacts with 50S subunits as well as 30S subunits by themselves and in 70S ribosomes and show that binding of the 50S subunit appears to cause a conformational change in the 30S subunit.

Materials and Methods

Materials

CPM, FM, and FTS were purchased from Molecular Probes, Inc. (Junction City, OR). Proteinase K, a product of E. Merck, was purchased through Beckman Instruments, Inc. (Irvine, CA). Guanidine hydrochloride, poly(U), folinic acid, and Sephadex products were from Sigma Chemical Co. (St. Louis, MO). Ultrapure urea and [^{14}C]methionine (260 Ci/mol) were from Schwarz/Mann (Spring Valley, NY). L-[^{14}C]Phenylalanine (460 Ci/mol) was obtained from ICN (Irvine, CA). MS2 RNA was a product of Boehringer Mannheim (Indianapolis, IN). All other chemicals were of reagent grade.

Solutions. TMNSH solution consisted of 10 mM Tris-HCl (pH 7.5), 10 mM Mg(OAc)$_2$, 100 mM NH_4Cl, and 5 mM β-mercaptoethanol. Solution A was 30 mM Tris-HCl (pH 7.5), 500 mM KCl, 20 mM Mg(OAc)$_2$, and 1 mM dithioerythritol.

Methods

Growth of E. coli Mutant. The designated *E. coli* mutant strain was isolated by E.R.D. This mutant has an altered protein S21 which is more readily removed from 30S subunits

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[1] Abbreviations: poly(S^{4}U), poly(4-thiouridylic acid); CPM, 3-(4-maleimidophenyl)-4-methyl-7-(diethylamino)coumarin; FTS, fluorescein thiosemicarbazide; FM, fluorescein-5-maleimide; CPM-S21, S21 labeled at its sulfhydryl group with CPM; FTS-16S RNA, 16S RNA labeled at its 3′ end with FTS; FM-L11, L11 labeled at its sulfhydryl group with FM; poly(U), poly(uridylic acid); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; 30S(−S1), 30S(−S21), and 30S(−S21, −S1), 30S subunits lacking the proteins indicated in parentheses; EDTA, ethylenediaminetetraacetic acid; EF-Tu, elongation factor Tu; BSA, bovine serum albumin.

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than is wild-type S21. The mutant was grown in the medium described by Dabbs (1980) in a 50-L fermenter at 35 °C. Harvesting of the cells by the continuous flow method was begun when the A_{650} of the culture reached 0.8.

Preparation of S21 and of S21-Deficient 30S Subunits. Homogeneous S21 was a kind gift of H. G. Wittmann (Max-Planck-Institut für Molekulare Genetik, Berlin). It was isolated by the acetic acid–urea method (Hindennach et al., 1971). Ribosomes were prepared from *E. coli* mutant cells as previously described (Odom et al., 1980). The procedure used for separation of subunits has been described in the same reference. To remove S21 from mutant 30S subunits, these were incubated in 1.5 M NH_4Cl, 10 mM $MgCl_2$, 20 mM Tris-HCl (pH 7.5), and 1 mM dithioerythritol at a concentration of 1 mg/mL for 3 h at 4 °C. Then the sample was layered over 5 mL of 20% sucrose in the same solution. The subunits were isolated by centrifugation for 15 h at 40000 rpm in a Spinco Ti 60 rotor. The 30S pellets were resuspended in TMNSH solution at a concentration of about 12 mg/mL. These washed 30S particles contained a small amount of residual S21, as evidenced from polyacrylamide gels run in the presence of urea under the conditions described by Kaltschmidt & Wittmann (1970) for the second dimension. This washing procedure also removes some of the S1, as was evident after electrophoresis of extracted 30S proteins on polyacrylamide gels, and by the fact that added S1 enhanced the activity of the washed 30S particles in poly(U)-dependent polyphenylalanine synthesis. Therefore, the washed 30S subunits were usually supplemented with S1 purified as previously described (Odom et al., 1984a).

Labeling of S21. The following procedure was used to label the sulfhydryl group of S21 with CPM. To ensure that no intermolecular disulfide bonds were present, the sample (at 1–5 mg/mL) was first incubated at 37 °C for 30 min in 7 M guanidine hydrochloride, 10 mM Hepes (pH 8.5), and 20 mM dithioerythritol. The latter compound was then removed by chromatography over Sephadex G25 in 7 M guanidine hydrochloride plus 10 mM Hepes (pH 7.5). To the S21 eluted from this column was added 0.01 volume of a 0.1 M solution of CPM in dimethylformamide. The resulting solution was incubated for 24 h on ice followed by 30 min at 37 °C, and then glutathione was added to give a final concentration of 10 mM to react with excess CPM reagent. This sample was rechromatographed over Sephadex G25 equilibrated with 7 M urea–20 mM Hepes (pH 7.5) to remove guanidine hydrochloride and excess labeling reagents. CPM-S21 eluted from the column was dialyzed against solution A and then stored in small aliquots at −80 °C.

For determining the stoichiometry of labeling, it was necessary to know both the concentration of bound CPM and the concentration of S21. The former was determined from its absorbance at its maximum, 398 nm. The molar extinction coefficient of bound CPM at this wavelength was determined to be 2.7×10^4 M^{-1} cm^{-1}. This determination was made by comparing the absorbance of CPM-S21 before and after treatment with 50 μg/mL proteinase K in solution A for 30 min at 37 °C. No further change in the absorbance of fluorescence occurred after 30 min or on addition of more proteinase K. It was assumed that the extinction coefficient after proteinase K treatment was equal to that of free CPM-cysteine in the same solution (determined to be 2.7×10^4 M^{-1} cm^{-1}). The S21 concentration was determined by the amidoschwarz method of Schaffner & Weissmann (1973) using bovine serum albumin as a standard. Using the above procedure, we calculated the stoichiometry of labeling to be about 0.9 mol of CPM per mol of S21.

Labeling of the 3′ End of 16S RNA. 16S RNA was oxidized with periodate and labeled with fluorescein 5′-thiosemicarbazide (FTS) as previously described (Odom et al., 1980).

Preparation of Initiation Factors. E. coli initiation factor fractions were prepared as described by Hershey et al. (1979). IF-2 was used after the ammonium sulfate fractionation step and was not subjected to ion-exchange chromatography. IF-1 and IF-3 were carried through the first phosphocellulose chromatography step.

Preparation of fMet-tRNA$_f$. fMet-tRNA$_f$ was prepared according to the method of Hershey & Thach (1967), except that an *E. coli* tRNA fraction containing only those tRNAs which elute from benzoylated DEAE-cellulose without ethanol was used following the fractionation procedure of Roy et al. (1971).

Reconstitution of Labeled S21 into 30S. The usual procedure for reconstituting S21 into 30S particles consisted of total reconstitution. This was as described previously (Odom et al., 1980) except that the ribosomal proteins used were extracted from 30S(−S21) and were supplemented with the stoichiometric amount of CPM-S21. For obtaining 30S particles containing both labeled S21 and labeled 16S RNA, we used FTS-16S RNA in place of unlabeled 16S RNA. Following reconstitution, the 30S subunits were isolated by sedimentation for 4 h at 49000 rpm in a Spinco Ti 50 rotor. The pellets were resuspended in TMNSH solution at a concentration of about 100 A_{260} units/mL.

In some experiments, such as assaying labeled S21 for activity, reconstitution of S21 into 30S particles was achieved simply by adding CPM-S21 to the 30S(−S21) in TMNSH solution, followed by incubation for 10 min at 37 °C. These particles were very similar in spectral characteristics and activity to totally reconstituted particles.

Activity of Labeled and Unlabeled Protein S21. Two methods were used to measure the activity of S21. One method consisted of the standard poly(U) assay described previously (Odom et al., 1980) modified by substituting 30S(−S21) with or without S21 for 30S. The other method measured MS2 RNA dependent binding of fMet-tRNA$_f$ to 30S subunits, essentially as described by van Duin & Wijnands (1981). More details of this method are given in the legend to Table I.

Purification and Labeling of Protein L11 and Reconstitution into 50S Subunits. Protein L11 was purified by a procedure involving extraction of 50S subunits with 2 M LiCl, (carboxymethyl)cellulose chromatography, and reverse-phase high-performance liquid chromatography. The details of the purification procedure are described elsewhere (H.-Y. Deng et al., unpublished results). Purified L11 was labeled with 1 mM FM in 10 mM Hepes–KOH (pH 7.5) and 7 M guanidine hydrochloride for 30 min at 37 °C, followed by chromatography on Sephadex G25 equilibrated with 10 mM Hepes–KOH (pH 7.5) and 7 M guanidine hydrochloride. Labeled L11 eluted from the G25 column was passed over a second G25 column equilibrated with 20 mM Tris-HCl (pH 7.5), 4 mM $Mg(OAc)_2$, 400 mM NH_4Cl, 2 mM β-mercaptoethanol, and 0.02 mM EDTA. The stoichiometry of labeling was near 1 mol of FM per mol of L11 (H.-Y. Deng et al., unpublished results).

A mutant of *E. coli* which is missing protein L11 (Dabbs, 1979), designated AM68, was supplied by E.D. It was grown in the medium described by Dabbs (1977) in a 50-L fermenter, and ribosomes and subunits were prepared as described pre-

viously (Odom et al., 1980). Labeled L11 was reconstituted into 50S(-L11) following the procedure of Nierhaus & Dohme (1979), using a 10% excess of FM-L11. Reconstituted particles were collected by sedimentation for 4 h in the Spinco Ti 50 rotor and resuspended in TMNSH solution.

Glycerol Gradient Sedimentation. The gradients contained 10–35% glycerol in 10 mM Tris-HCl (pH 7.5), 20 mM $Mg(OAc)_2$, 100 mM NH_4Cl, and 6 mM β-mercaptoethanol. They were formed in Spinco SW 41 tubes by using the Isco Model 579 gradient former (Instrumentation Specialties, Lincoln, NB). Samples to be sedimented were layered on the gradients in a volume of 0.3 mL. The gradients were centrifuged as indicated.

Fluorescence Measurements. Fluorescence measurements were taken with an SLM Model 8000 photon counting spectrofluorometer (SLM Instruments, Inc., Urbana, IL) as described previously (Odom et al., 1980).

Steady-state fluorescence polarization and anisotropy measurements were made with the SLM fluorometer by using polarizers in the excitation and emission light paths. The following designations are used to indicate whether the orientations of the polarizers are vertical (v) or horizontal (h): *vv*, *vh*, *hv*, and *hh*, the first letter referring to the excitation polarizer and the second letter to the emission polarizer. Polarization (P) is defined as

$$P = \frac{vv - vh}{vv + vh}$$

and anisotropy (A) as

$$A = \frac{vv - vh}{vv + 2vh}$$

A correlation factor for disparity in the efficiency of counting vertical and horizontal polarized light was determined by orienting the excitation polarizer horizontally. In this orientation, the intensity of light passing through the emission polarizer should be the same whether it is oriented vertically or horizontally, since both orientations are orthogonal to that of the excitation polarizer. Therefore, any observed differences in intensity are due to differences in sensitivity of the photomultiplier, and the correction factor for *vh* in the above equations is $hv/(hh)$.

The quantum yield of CPM-S21, reconstituted into 30S subunits, was determined by integrating its emission spectrum and comparing it with the integrated emission spectrum of quinine sulfate, which has a quantum yield of 0.70 (Scott et al., 1970). Excitation of both samples was at 360 nm, and the absorbance of the two samples was made 0.0065 by suitable dilution of concentrated stock solutions.

Energy transfer calculations were made as previously described (Odom et al., 1980), by using fluorescence intensity measurements of the fluorescence donor in the absence and presence of the fluorescence acceptor. The existence of energy transfer was also usually checked by measuring the enhancement of fluorescence of the energy acceptor in the presence of donor.

In order to correct for any differences in concentration, between the singly and doubly labeled samples used for energy transfer, we added $^1/_9$th volume of 15 M LiCl to the samples after the fluorescence measurements. The resulting final concentration of 1.5 M LiCl causes several proteins including S21 to dissociate from the 30S subunit (Homann & Nierhaus, 1971). Thus, energy transfer is eliminated, and fluorescence is proportional to the concentration of the fluorophore. All samples used for measuring energy transfer were normalized to equal concentration by this method.

Table I: Activity of CPM-S21 in MS2 RNA Directed Binding of $fMet\text{-}tRNA_f$ to 30S Subunits[a]

S21 added	fMet-tRNA bound (cpm)	
	unmodified S21	CPM-S21
none	251	251
0.05 μg	361	375
0.10 μg	439	511
0.28 μg	520	565
native 30S[b]	521	

[a] Reaction mixtures in 100 μL contained 40 mM Tris-HCl (pH 7.5), 60 mM NH_4Cl, 7 mM $Mg(OAc)_2$, 6 mM β-mercaptoethanol, 1 mM GTP, 32 pmol of [^{14}C]$fMet\text{-}tRNA_f$ (260 Ci/mol), 0.3 A_{260} unit of 30S(-S21) subunits or unmodified 30S containing native S21, optimal amounts of IF-1, IF-2, and IF-3, and 0.33 A_{260} unit of MS2 RNA. Incubation was for 10 min at 37 °C followed by cooling on ice and filtration through nitrocellulose filters. A sample lacking MS2 RNA gave 239 cpm. This has been subtracted from the values given above. [b] No additional S21 added.

Results

Activity of CPM-S21. The activity of unlabeled S21 and CPM-S21 for binding of [^{14}C]$fMet\text{-}tRNA_f$ to 30S(-S21) with MS2 RNA is shown in Table I. Approximately the same level of maximum binding is reached with either labeled or unmodified wild-type S21 as is obtained with unmodified 30S subunits containing native S21. However, about 50% of the maximum binding of $fMet\text{-}tRNA_f$ is observed in the absence of added S21. This may be due in part to a small amount of mutant S21 remaining in the preparation of S21-deficient 30S subunits.

It should be noted that the molar amount of $fMet\text{-}tRNA_f$ bound is less than 10% of the molar amount of ribosomes present in the reaction mixture. This is in agreement with the results of van Duin & Wijnands (1981). Also, these authors reported no effect of added S21 on poly(U)-dependent polyphenylalanine synthesis with ribosomes reconstituted in the absence of S21. However, we obtain a 25–55% increase in polyphenylalanine synthesis with S21, added either during total reconstitution or after reconstitution. This agrees with the results of Held et al. (1974). CPM-S21 and unlabeled S21 are equally active in giving this increase (Odom et al., 1984b).

Spectral Properties of CPM-S21. The emission maximum of CPM-S21 is at 478 nm in the aqueous solvent used for the experiments of Table II. Coumarin is an environmentally sensitive probe. The relatively short wavelength of this maximum suggests that the coumarin fluorophore is in a hydrophobic environment within the S21 protein. For comparison, CPM-cysteine exhibits a fluorescence maximum at 486 nm in the solvent used but a maximum at 470 nm in 95% ethanol. The quantum yield of CPM-cysteine is increased by a factor of 1.9 in the latter solvent. On incorporation into 30S subunits, the CPM-S21 emission maximum shifts from 478 to 468 nm with about a 50% increase in quantum yield. The results of Table II suggest that the local environment of the fluorophore becomes even more hydrophobic when the protein is associated with a 30S ribosomal subunit. Also, the static anisotropy of CPM-S21 increases from 0.20 for the free protein to 0.35 in 30S subunits. The latter value indicates a high degree of immobilization of the coumarin moiety, when the labeled protein is associated with the ribosomal subunit. Addition of 50S subunits to free CPM-S21 also causes a change in the emission maximum, relative intensity of fluorescence, and anisotropy, reflecting binding of S21 to this subunit as described below.

Addition of 50S subunits to the CPM-S21·30S complex causes a small additional increase in fluorescence intensity with no change in the emission maximum. LiCl at a concentration

Table II: Spectral Properties of CPM-S21 under Various Experimental Conditions[a]

exptl conditions[b]	fluorescence λ_{max}	fluorescence rel intensity[c]	fluorescence anisotropy
CPM-S21 only	478	0.64	0.20
+30S(-S21)	468	1.0	0.35
+30S(-S21) + 50S	468	1.06	0.36
+50S	468	1.13	0.35
+16S RNA	478	0.65	0.20
+1.5 M LiCl	478	0.61	0.20
+30S(-S21) + 1.5 M LiCl	478	0.61	0.20

[a] The emission maximum of CPM-cysteine in the solvent used for CPM-S21 is at 486 nm but at 470 nm in 95% ethanol. The quantum yield is 1.9-fold higher in the latter solvent. The absorbance maximum of free or 30S-bound CPM-S21 in TMNSH solution is at 398 nm, compared to 393 nm for free CPM-cysteine in the same solution. The molar extinction coefficient at the absorbance maximum is 2.7×10^4 for free CPM-S21, 2.5×10^4 for 30S-bound CPM-S21, and 2.7×10^4 for free CPM-cysteine. [b] All samples were in TMNSH solution containing CPM-S21 plus the other components indicated. 30S(-S21) and 50S subunits were added to give a molar ratio of 5 with CPM-S21 where indicated. [c] Fluorescence intensities are relative to CPM-S21 + 30S(-S21) subunits, which have been arbitrarily assigned a value of 1.0.

of 1.5 M causes many ribosomal proteins including S21 to dissociate from the ribosomal complex (Homann & Nierhaus, 1971). It is used for this purpose in some of the experiments described below to increase the distance between the fluorophore on S21 and an energy acceptor probe so that nonradiative energy transfer is eliminated. The effect of 1.5 M LiCl on the fluorescence from CPM-S21 free in solution and with 30S subunits also is shown in Table II. With both samples, 1.5 M LiCl reduces the relative intensity and anisotropy to the same values, 0.61 and 0.20, respectively. These results demonstrate that the salt causes dissociation of CPM-S21 under the experimental conditions used.

Interaction of CPM-S21 with 50S Subunits. The data of Table II indicate a sharp increase in the anisotropy of fluorescence from CPM-S21, when either 30S or 50S subunits are added to a solution containing the labeled protein. This increase in anisotropy appears to reflect a decrease in the rate of movement of the labeled protein that occurs when it binds to the relatively large subunits. The rotational correlation time, τ, for free S21 is less than 10 ns, whereas this value for either of the ribosomal subunits is more than 1 μs. Thus, changes in anisotropy provide a sensitive measure for binding of the protein to the ribosomal subunits. This phenomenon was used for the experiments represented by Figure 1 in which the steady-state polarization of fluorescence from an 83 nM solution of CPM-S21 was measured with increasing amounts of added ribosomal subunits. The results are plotted as the molar ratio of ribosomal subunits to CPM-S21. An apparently linear increase in anisotropy from 0.20 to about 0.31 was observed with increasing amounts of 30S subunits to a 30S/CPM-S21 ratio of about 0.85. We interpret this to reflect tight binding of the protein to a single site on the 30S subunit. The basis for the molar ratio of 0.85 rather than 1.0 is not clear. Also, the cause of the further increase in anisotropy from 0.31 to a maximum of about 0.35 with larger amounts of subunits (see Table II) is not clear. This increase may reflect an interaction between 30S subunits at higher subunit concentrations that affects the depolarization of CPM-S21 fluorescence.

In contrast to the results with 30S subunits, the 50S subunits cause a linear increase in anisotropy to 0.31 at a 50S/CPM-S21 ratio of about 0.44. This may reflect binding of CPM-S21 to two sites on the 50S subunits. Further addition of 50S

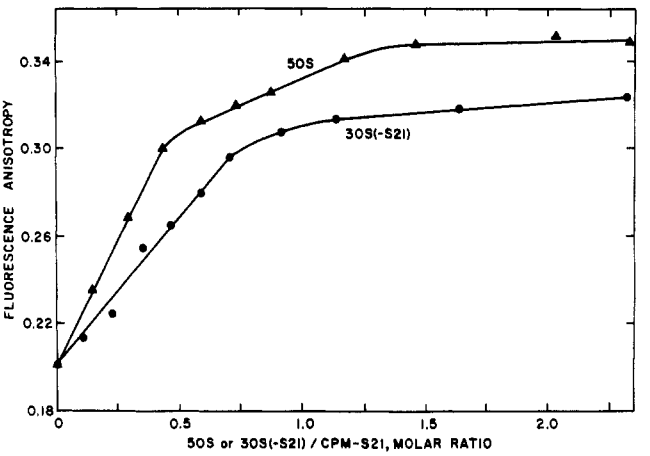

FIGURE 1: Subunit saturation curves for binding of CPM-S21 to 30S(-S21) or 50S subunits. Samples contained 50 pmol of CPM-S21 and the indicated amount of 30S(-S21) or 50S subunits in 0.6 mL of TMNSH solution. Measurements of anisotropy were made at 30 °C as described under Methods. (●) 30S(-S21); (▲) 50S subunits.

subunits causes an additional increase in anisotropy to a maximum value of near 0.35. The portion of the curve between ribosome/CPM-S21 ratios of 0.45 to at least 0.9 is apparently linear as judged from repeated experiments. Although we are well aware of the hazards of overinterpretation of such apparently biphasic curves, we suggest that this portion of the curve may reflect redistribution of CPM-S21 between a weak and a strong binding site which gives fluorescence anisotropy of about 0.27 and 0.35, respectively. Presumably, the distribution of CPM-S21 is shifted toward a considerably stronger binding site at higher concentrations of 50S subunits. CPM-glutathione and CPM-cysteine were used in place of CPM-S21 in experiments comparable to those described above with 50S subunits. Neither compound bound significantly to 50S subunits as judged by their fluorescence spectra and anisotropy. These results suggest that CPM-S21 binding is not due to strong binding of the CPM moiety.

Relative Affinity of CPM-S21 for 30S or 50S Subunits and 70S Ribosomes. The data of Figure 1 indicate that at 83 nM very little CPM-S21 exists free in solution in the presence of a molar excess of either 30S or 50S subunits. Thus, the dissociation constant must be appreciably below this value. However, attempts to measure dissociation constants for 50S and 30S subunits directly by fluorescence techniques have not been successful, because the value of the constants appears to be below the concentration of CPM-S21 that can be accurately measured in our system.

Analysis by glycerol density gradient centrifugation was used to test the relative affinity of CPM-S21 for free 30S or 50S subunits and for 70S ribosomes. In one set of experiments, a 1.5-fold molar excess of 50S subunits was added to CPM-S21 free in solution, and then the resulting mixture containing CPM-S21·50S subunits was centrifuged in the absence or presence of a 2-fold molar excess of 30S subunits. The results are shown in Figure 2. Fluorescence and absorption at 260 nm were coincident at the 50S position for CPM-S21·50S alone, but in the presence of excess 30S subunits, at least 90% of the 50S subunits and most of the fluorescence were in the 70S position. Relatively little fluorescence was present with free 30S subunits. This somewhat surprising result appears to indicate that CPM-S21 binds more tightly to 70S ribosomes than to 30S subunits. The CPM-S21·30S complex was analyzed by gradient centrifugation in experiments similar to those carried out with the CPM-S21·50S complex. A 1.4-fold molar excess of 30S subunits was added to CPM-S21 free in solution.

Table III: Binding of CPM-S21 to 70S vs. 50S and to 70S vs. 30S Ribosomes[a]

conditions	Fl/mol of 50S	Fl/mol of 30S	Fl/mol of 70S	(Fl/mol of 70S)/ (Fl/mol of 50S)	(Fl/mol of 70S)/ (Fl/mol of 30S)
excess 30S		1094	3602		3.3
excess 50S	<200		4504	>20	

[a] Numbers given are calculated from the data of Figure 2 and a similar experiment carried out with CPM-S21·30S alone or in the presence of 50S subunits. Fl/mol means the relative fluorescence intensity per mole of ribosomes. This is essentially proportional to the amount of CPM-S21 per mole, since the quantum yields of CPM-S21 in the 30S, 50S, and 70S complexes do not differ greatly, as shown in Tables IV and V.

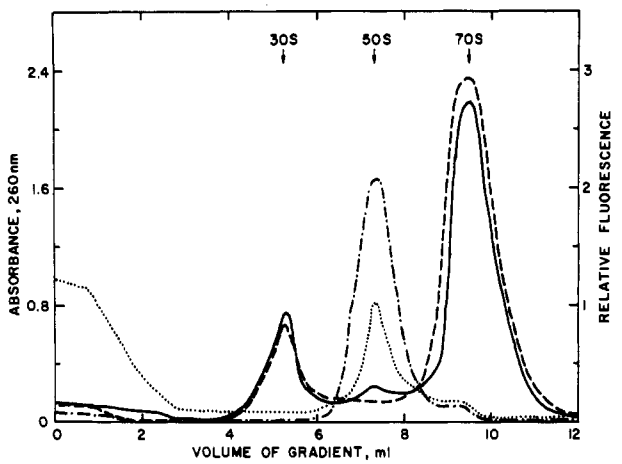

FIGURE 2: Glycerol gradient profile of CPM-S21 with 50S alone or in the presence of an excess of 30S(-S21). 78 pmol (2 A_{260} units) of 50S subunits in a volume of 0.3 mL was incubated with 50 pmol of CPM-S21 for 5 min at 42 °C in TMNSH solution. Then 160 pmol (2.3 A_{260} units) of 30S(-S21) was added to one sample and incubation continued for 5 min at 37 °C. The samples with and without 30S were then layered on gradients and sedimented for 16 h at 23 000 rpm in the SW 41 rotor. (-·-) A_{260} of CPM-S21 + 50S alone; (···) fluorescence of CPM-S21 and 50S alone; (—) A_{260} of CPM-S21 + 50S + excess 30S(-S21); (- - -) fluorescence of CPM-S21 + 50S + excess 30S(-S21).

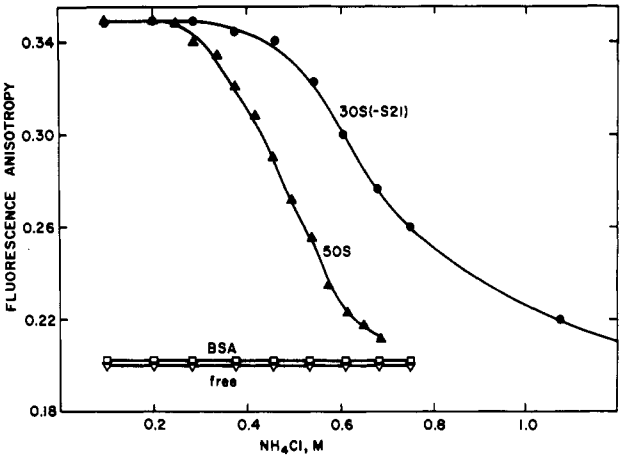

FIGURE 3: Comparison of the stability of the 30S and 50S complexes of CPM-S21 as a function of NH_4Cl concentration. Samples contained 60 pmol of CPM-S21 with a 5-fold molar excess of either 30S(-S21) or 50S. The sample with serum albumin contained 0.6 mg/mL albumin. Anisotropy measurements were made at 30 °C as described under Methods. (●) +30S(-S21); (▲) +50S; (□) BSA; (△) free CPM-S21.

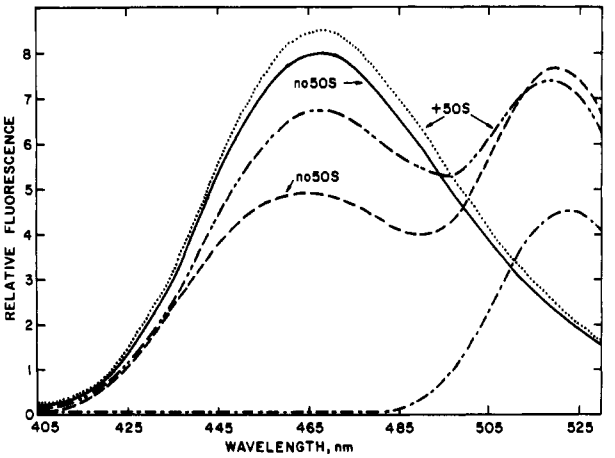

FIGURE 4: Emission spectra of doubly and singly labeled reconstituted 30S with and without added 50S. Samples contained 1 A_{260} unit of reconstituted 30S in 0.60 mL of TMNSH solution. After the spectra were taken without 50S, 2.0 A_{260} units of 50S were added, and the spectra were retaken. Corrections have been made for background fluorescence, dilution, and concentration differences between samples (by the LiCl method described under Methods), so that the intensities shown are proportional to quantum yields. (—) CPM-S21·unlabeled 16S·30S; (- - -) CPM-S21·FTS-16S·30S; (···) CPM-S21·unlabeled 16S·30S + 50S; (-··-) CPM-S21·FTS-16S·30S + 50S; (-·-) unlabeled S21·FTS-16S·30S ± 50S.

The resulting mixture containing the CPM-S21·30S complex was then centrifuged in the absence or presence of a 3-fold molar excess of 50S subunits to the total amount of 30 S present and analyzed as described above. Fluorescence and absorption at 260 nm were coincident at the 30S position for the CPM-S21·30S complex alone, but in the presence of excess 50S subunits, at least 90% of the 30 S and nearly all of the fluorescence were found in the 70S position. This result demonstrates that CPM-S21 binds much more tightly to 70S ribosomes than to 50S subunits.

The results described above are quantitatively summarized in Table III. The results demonstrate that CPM-S21 initially bound to either 30S or 50S subunits is not appreciably redistributed to the other subunit, when it is present in molar excess, but rather it binds preferentially to 70S ribosomes. The molar ratio of binding to 70S ribosomes vs. 50S subunits is greater than 20. These data appear to indicate that the relative affinity of CPM-S21 is 70 S > 30 S > 50 S under the experimental conditions used. They suggest that each of the subunits contributes to binding S21 on the 70S ribosome.

Dissociation of CPM-S21 from 30S or 50S Subunits by NH_4Cl. The effects of NH_4Cl on binding of CPM-S21 to either 30S or 50S subunits were measured as an indication of the relative affinity of the protein for the two subunits. Under the conditions used, higher concentrations of monovalent salts such as LiCl or NH_4Cl cause many ribosomal proteins including S21 to dissociate from ribosomal subunits. Dissociation of CPM-S21 from 50S and 30S subunits with increasing concentrations of NH_4Cl was followed by changes in anisotropy in experiments similar to those described above in Table II.

The results, shown in Figure 3, indicate that the complex formed with 30S subunits is stable in higher concentrations of salt than is the complex formed with 50S subunits. Half-maximal decreases in anisotropy occur with 0.49 and 0.71 M NH_4Cl for dissociation from 50S and 30S subunits, respectively. By this criterion, binding is relatively strong to both subunits, but stronger to the 30S subunit.

Energy Transfer between CPM-S21 and FTS-16S RNA in 30S Subunits. Figure 4 shows emission spectra of CPM-

Table IV: Energy Transfer between CPM-S21 and FTS-16S RNA in 30S and 70S Ribosomes

conditions	Q	E^a (%)	R_0 (Å)	r' (Å)^b	half-height limits^b of $Q(r'/r)$	limits of r (Å)^b
30S alone	0.78	49	51	51	0.88–1.15	44–58
30S + 50S	0.83	27	52	61	0.88–1.15	53–69

[a] The observed percent energy transfer is calculated from the quenching of the donor fluorescence and has been corrected for the fact that only 80% of the ribosomes contain the fluorescein acceptor. [b] The half-height limits were calculated according to the method of Haas et al. (1978) using polarization values of 0.45 for CPM and 0.32 for fluorescein. The distance calculated assuming the orientation factor (κ^2) is equal to $^2/_3$ is called r'; r is the actual distance.

Table V: Energy Transfer between CPM-S21 and FM-L11 in 50S and 70S Ribosomes

conditions	Q	E^a (%)	R_0 (Å)	r' (Å)^b	half-height limits^b of $Q(r'/r)$	limits of r (Å)^b
50S alone	0.88	8.4	52	77	0.88–1.15	67–88
50S + 30S	0.83	8.9	52	77	0.88–1.15	67–88

[a] Calculated from the quenching of donor (CPM-S21) fluorescence. [b] Calculated as described in Table III, using polarization values of 0.45 for CPM and 0.32 for fluorescein.

S21·30S, CPM-S21·FTS-16S RNA·30S, and FTS-16S RNA·30S. The spectra were normalized to equal concentrations by the LiCl procedure described under Methods. Spectra were taken, then LiCl to give 1.5 M concentration was added to each sample, and the spectra were retaken. Energy transfer between the probes is eliminated by the salt so that the intensity of fluorescence is proportional to the concentration of CPM-S21 in the samples. Comparison of the spectra of CPM-S21·30S and CPM-S21·FTS-16S RNA·30S of Figure 4 indicates a 39% decrease in the intensity of fluorescence from the energy donor, coumarin, in the doubly labeled sample. This is due to energy transfer from the coumarin moiety of CPM-S21 to the fluorescein moiety of FTS at the 3′ end of the 16S RNA.

The requisite parameters and distances between the probes, calculated from energy transfer, are given in Table IV. The calculated distance between the probes of CPM-S21 and FTS-16S RNA is 51 Å. This includes a correction for unpaired donor. About 80% of the ribosomes contain the acceptor, FTS, on 16S RNA. The fluorescence of the acceptor also is enhanced in the doubly labeled sample, which confirms energy transfer. Since both donor and acceptor probes have rather high fluorescence anisotropies, the range of the probable distance, calculated according to the method of Haas et al. (1978), is rather large. It should be noted that these limits reflect uncertainty in the value for κ^2, the factor for the orientation of the probes, and not error in the determination itself.

Effect of 50S on Energy Transfer between CPM-S21 and FTS-16S RNA in 30S Subunits. The data of Figure 4 indicate that 50S subunits cause an increase in the intensity of coumarin fluorescence with either CPM-S21·30S or CPM-S21·FTS-16S RNA·30S. However, the percentage of increase is larger for the doubly labeled sample. As shown in Table IV, the calculated percent energy transfer decreases from 49% to 27%, when an excess of 50S subunits is added to the sample containing doubly labeled 30S subunits. A concomitant decrease in fluorescence from fluorescein, the energy acceptor, occurs, confirming the conclusion that the 50S subunits have caused a decrease in energy transfer. The change in energy transfer corresponds to a change in the distance between the probes from 51 to 61 Å.

Energy transfer between CPM-S21 and FTS-16S RNA also was measured in 30S subunits and 70S ribosomes taken from glycerol gradients comparable to those represented by Figure 2. Samples from the 30S and 70S positions show 50% and 29% energy transfer, respectively, that is, in the absence and presence of 50S subunits. These values are very similar to those given in Table IV. The data appear to indicate that CPM-S21 remains fixed in a binding site on a 30S ribosome during formation of a 70S complex since it is unlikely that S21 would be repositioned in the 70S ribosome into an entirely different site in which its distance from the 3′ end of 16S RNA was within 10 Å of this distance in 30S subunits. However, these data do indicate that binding of the 50S subunit causes a change in energy transfer that is consistent with an increase in the distance between probes on S21 and the 3′ end of 16S RNA by about 10 Å.

Energy Transfer between CPM-S21 and FM-L11 on 50S Subunits. Energy transfer experiments comparable to those described above for the 30S subunit were carried out by using fluorescein-labeled L11 (FM-L11) on the 50S subunit. L11 contains a single cysteine residue that was labeled by reaction with fluoresceinmaleimide as described under Methods. The ratio of fluorescein to L11 was 1:1, within the accuracy of our determinations. FM-L11 was added to 50S subunits that were isolated from a mutant strain of *E. coli* that completely lacks L11 [cf. Dabbs (1979)]. CPM-S21 was added to these 50S subunits and energy transfer measured in the absence and presence of a 1.5-fold molar excess of 30S subunits. The results are presented in Table V. About 8.4% energy transfer, corresponding to a distance of 77 Å between the probes, was measured with 50S subunits only. Energy transfer of 8.9%, corresponding to 77 Å, was measured in the presence of 30S subunits. This is not a significant difference in energy transfer under the experimental conditions used. Energy transfers of 9% and 13% were measured in the 50S peak and 70S peak, respectively, from glycerol gradients comparable to those represented by Figure 2.

Discussion

The results shown above demonstrate conclusively that CPM-S21 binds tightly to 50S as well as to 30S ribosomal subunits. Although they must be interpreted with caution in this regard, the data of Figure 1 suggest that there is one very tight binding site on the 30S subunit and two sites on the 50S subunit, one of which gives considerably more stable binding than the other. The critical question involves the significance of S21 binding to the 50S subunit. Is this an artifact of the in vitro system or does it occur in vivo? We know of no previous report of this phenomenon and find it surprising that it has not been detected in earlier studies. Part of the explanation may involve the relative affinity of the protein for the two subunits. Under most experimental conditions involving subunit dissociation, it appears that the protein either would remain bound to the 30S subunit or be released free in solution. The question of the functional significance and the number of CPM-S21 binding sites on the 50S subunit cannot be resolved by the data presented here, and we rec-

ommend caution in their interpretation in this regard. However, the data do provide an indication that S21 may interact simultaneously at the tight binding sites on both the 30S and 50S subunits within the 70S ribosome. The results from density gradient centrifugation experiments, represented by Figure 2 and Table III, demonstrate that CPM-S21 is bound more tightly to 70S ribosomes than to either subunit separately. That is, both subunits appear to contribute to the stability of the S21·70S ribosome complex. Furthermore, the data of Tables IV and V demonstrate that energy transfer from CPM-S21 to FTS-16S RNA in 30S subunits or from CPM-S21 to FM-L11 in 50S subunits does not change a large amount (49% to 27% for the former) on binding of the other subunit to form 70S ribosomes. These data appear to indicate that CPM-S21 molecules involved in energy transfer are in about the same position in isolated subunits as they are in 70S ribosomes. Considering the apparent difference in the binding constants of CPM-S21 for 30S vs. 50S subunits, it seems likely that CPM-S21 bound initially to 50S subunits would redistribute to the stronger binding site on 30S subunits in 70S ribosomes, if two physically distinct and independent sites were involved. If redistribution to the 30S binding site occurred, it is likely that energy transfer to FM-L11 would change. The CPM-S21 to FM-L11 distance was calculated to be 77 Å for both 50S subunits and 70S ribosomes.

Binding of the 50S subunit does cause a change in energy transfer from 49% to 27%, corresponding to a change of about 10 Å in the distance between CPM-S21 and FTS-16S RNA in the 30S subunit. However, it cannot be concluded from the data presented here that the measured change in energy transfer is due exclusively to a change only in distance. The change may be partly due to a change in the relative orientation of the probes that results in a change in the true value of κ^2, the orientation factor. A change in the relative orientation of the probes leading to the observed change in energy transfer might be due to a change in the conformation of the 30S subunit to which the probes are attached or to a direct effect of the 50S subunits on the probes themselves. The latter is unlikely. Coumarin fluorescence from CPM-S21 is very sensitive to the local environment of the probe. A direct interaction of the 50S subunit with this probe would likely cause relatively large changes in its quantum yield, emission maximum, or anisotropy. The data of Table II show that such changes do not occur. A similar analysis has been made for the energy acceptor probe, FTS-16S RNA, by using direct excitation of the fluorescein probe. Addition of 50S subunits caused a 9% increase in relative fluorescence and a 9% decrease in anisotropy (unpublished results). A direct steric effect of the 50S subunits on the probe itself probably would further constrain its movement and increase rather than decrease its polarization. Furthermore, even though fluorescein fluorescence is less sensitive to perturbation by most environmental changes than fluorescence from coumarin, a direct interaction of the 50S subunit with the probe probably would have a larger effect on the relative fluorescence intensity than that observed. Thus, it appears likely that binding of a 50S subunit causes a conformational change in the 30S subunit that changes the position of the 3′ end of 16S RNA. It cannot be concluded what portion of the change in energy transfer is due to a change in probe orientation vs. distance. However, the heteropolar distribution of the electronic state of the probes used tends to minimize the effect of a change in probe orientation, as considered in detail by Haas et al. (1978), making it likely that the major effect is a change in distance. In this regard, it should be noted that the limits given for the calculated distance involve uncertainty about the value of κ^2 and are not a measure of the error present in the measurement of energy transfer. This error is much smaller than the 22% change we have observed.

A change in the physical relation of S21 and the 3′ end of 16S RNA is likely to account for the observation of Chiam & Wagner (1983) that S21 was not cross-linked to 16S RNA in 70S ribosomes. Also, it may be related to the observation of Zamir and her co-workers that the cysteine sulfhydryl group of S21 was reactive in 70S ribosomes but not in 30S subunits (Ginzberg & Zamir, 1975). In addition, Huang & Cantor (1972) reported that S21 was more reactive on 70S ribosomes than on 30S subunits with the amino group specific reagent fluorescein isothiocyanate.

Although to our knowledge S21 has never been cross-linked to any components of the 50S subunit, there have been some earlier indications that it is located near the interface between the subunits. Thus, when 30S subunits were preincubated with Fab antibody fragments against S21, EF-Tu-catalyzed Phe-tRNA binding was more strongly inhibited than when 70S ribosomes were treated, suggesting that the antibody inhibited subunit association (Lelong et al., 1974). The anti-S21 Fab fragment also abolished protection of bound Phe-tRNA from pancreatic RNase digestion, again indicating that it prevented formation of 70S couples (Stöffler, 1974).

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Antibody Inhibition of Ferripyochelin Binding to *Pseudomonas aeruginosa* Cell Envelopes†

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ABSTRACT: A 14K molecular weight protein which has been shown to bind ferripyochelin has been purified from cell envelopes of *Pseudomonas aeruginosa* low iron grown cells. The purified protein migrated as a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and was shown to be free of contamination by lipopolysaccharide or carbohydrate. Antiserum to this protein was made in rabbits and was shown to react with the purified protein by immunoblot assay. The immunoglobulin G fraction of this antiserum blocked binding of [^{59}Fe]pyochelin to isolated cell envelopes of *P. aeruginosa* in a dose-dependent fashion.

Iron is an essential requirement for the establishment and maintenance of bacterial infections (Bullen et al., 1978); Payne & Finkelstein, 1978; Weinberg, 1978). Since iron is either insoluble or complexed with host iron binding proteins, bacteria must possess iron sequestration and transport systems in order to compete for available iron (Neilands, 1981).

Most microorganisms excrete highly specific iron chelators, termed siderophores, which are taken up by cells in complex with Fe^{III} ion (Neilands, 1981). In many bacteria, proteins in the outer membrane have been determined to act as specific receptors for these iron–siderophore complexes. The function of these receptors is to bring the ferrisiderophore to, or through, the envelope where the iron undergoes a reductive separation from the chelator (Neilands, 1982).

A number of siderophore systems have been described for *Pseudomonas aeruginosa*; however, little is understood about the mechanism of iron transport in this organism (Neilands, 1982). Pyochelin is probably the most well-characterized siderophore of *P. aeruginosa*. Pyochelin is a salicylic acid substituted cysteinyl peptide (Cox et al., 1981). Uptake of label from [^{55}Fe]pyochelin has been shown to occur in two stages, an energy-independent step, where it presumably binds to the cell surface, followed by an energy-dependent process (Cox, 1980). We have recently identified a cell envelope of *P. aeruginosa* which binds [^{59}Fe]pyochelin (Sokol & Woods, 1983). This protein has a molecular weight of 14 000 and is produced in high concentrations in iron-starved glucose-grown cells. This protein was shown to bind [^{59}Fe]pyochelin but not $^{59}FeCl_3$. This protein is produced by all strains of *P. aeruginosa* examined, as well as several other species of *Pseudomonas* (Sokol, 1984).

In this paper, we describe the purification of this ferripyochelin binding protein and the production of antibody to the purified protein. Further, the ability of this antibody to

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