Published online October 27, 2004

5750–5756 *Nucleic Acids Research, 2004, Vol. 32, No. 19*
doi:10.1093/nar/gkh913

Multiple defects in translation associated with altered ribosomal protein L4

Michael O'Connor*, Steven T. Gregory[1] and Albert E. Dahlberg[1]

School of Biological Sciences, University of Missouri-Kansas City, 5007 Rockhill Road, Kansas City, MO 64110, USA and [1]J. W. Wilson Laboratory, Department of Molecular and Cellular Biology and Biochemistry, Brown University, Providence, RI 02912, USA

Received September 7, 2004; Revised and Accepted October 8, 2004

ABSTRACT

The ribosomal proteins L4 and L22 form part of the peptide exit tunnel in the large ribosomal subunit. In *Escherichia coli*, alterations in either of these proteins can confer resistance to the macrolide antibiotic, erythromycin. The structures of the 30S as well as the 50S subunits from each antibiotic resistant mutant differ from wild type in distinct ways and L4 mutant ribosomes have decreased peptide bond-forming activity. Our analyses of the decoding properties of both mutants show that ribosomes carrying the altered L4 protein support increased levels of frameshifting, missense decoding and readthrough of stop codons during the elongation phase of protein synthesis and stimulate utilization of non-AUG codons and mutant initiator tRNAs at initiation. L4 mutant ribosomes are also altered in their interactions with a range of 30S-targeted antibiotics. In contrast, the L22 mutant is relatively unaffected in both decoding activities and antibiotic interactions. These results suggest that mutations in the large subunit protein L4 not only alter the structure of the 50S subunit, but upon subunit association, also affect the structure and function of the 30S subunit.

INTRODUCTION

Accurate decoding of mRNA is a prerequisite for the transfer of information from DNA to proteins. The measured error rate of misincorporation during translation *in vivo* is c. 10^{-4} (1,2). However, genetic analyses in bacteria, yeast and filamentous fungi have led to the isolation of mutants that support either higher or lower levels of translational errors (2–5). Biochemical characterization of these mutants has revealed the identity of some of the protein synthesis components that are inferred to interact with tRNAs and mRNA on the ribosome and ensure faithful decoding. Recent X-ray crystallographic studies have shown that many of the rRNA residues and ribosomal proteins that are known from genetic analyses to be involved in decoding are at, or close to the decoding center on the 30S ribosomal subunit (6,7). While decoding occurs on the 30S subunit and is influenced by antibiotics, alterations to 16S rRNA and 30S ribosomal proteins, the genetic approaches described above have also uncovered a variety of mutations in 50S ribosomal proteins and 23S and 5S rRNAs that modulate the accuracy of decoding (2,8–14). The X-ray crystal structures of 50S subunits and 70S ribosomes have shown that at least some of these mutant 50S components have the potential to alter the interaction of the 50S subunit with tRNAs and elongation factors (15,16). Together these results indicate that interaction of regions of a tRNA outside of the anticodon with the ribosome can also influence the decoding step.

Antibiotic inhibitors of protein synthesis have served as useful probes of ribosome function and several mutations affecting translational accuracy have been identified in selections for antibiotic resistant mutants. Erythromycin binds at the top of the peptide exit channel in the 50S subunit and halts protein synthesis by blocking the nascent peptide's access to the tunnel (17,18). Structural analyses have shown that proteins L4 and L22 surround the otherwise RNA-rich tunnel (Figure 1). Erythromycin resistant ribosomes can arise through modification of rRNA, mutation of ribosomal proteins L4 and L22 or certain rRNA residues that comprise the protein exit tunnel (19). In *Escherichia coli*, resistant strains carrying either a Lys63Glu change in protein L4 or deletion of amino acids 82–84 in L22 have been described previously (20,21). Biochemical studies have shown that ribosomes containing the mutant L4 protein no longer bind erythromycin and are defective in peptide bond formation. In contrast, ribosomes carrying the altered L22 protein still bind the antibiotic and have unaltered peptidyltransferase activity (20). Subsequent cryo-EM reconstructions indicated that the L4 mutant protein caused a narrowing of the entry of the tunnel that apparently prevented erythromycin binding while the altered L22 caused a widening of the tunnel that allowed drug binding without inhibiting entry of the nascent peptide into the enlarged tunnel. In addition to effects on the erythromycin binding site, this study also showed that each mutation causes extensive alterations to the conformation of the 50S subunit (22,23). Moreover, the conformational effects of L4 and L22 mutations extended across the subunit interface, resulting in changes in the 30S subunit conformation. These observations raised the possibility that either or both of these ribosomal protein mutations might affect 30S functions, including the accuracy of tRNA selection.

*To whom correspondence should be addressed. Tel: +1 816 235 6372; Fax: +1 816 235 5595; Email: oconnormi@umkc.edu

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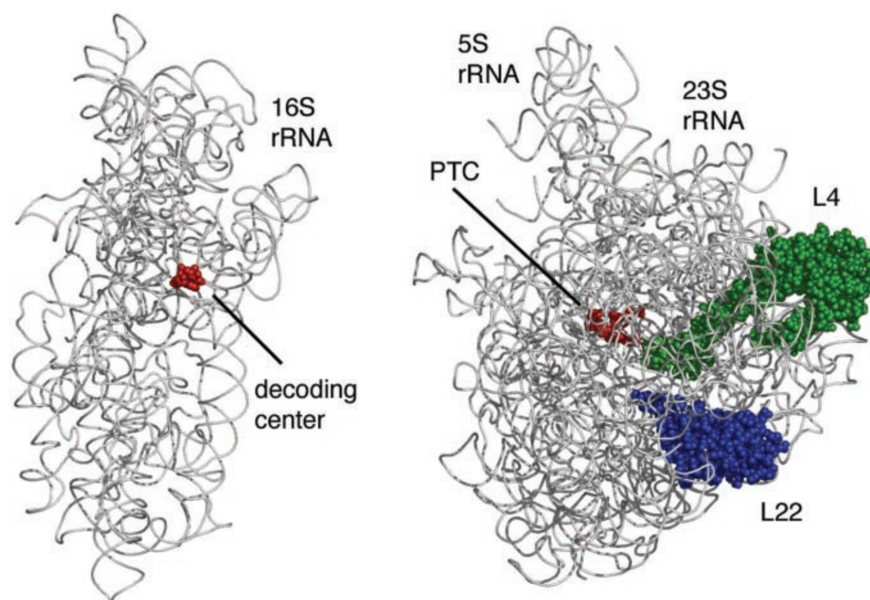

Figure 1. Location of ribosomal proteins L4 and L22 in the 50S ribosomal subunit of *Haloarcula marismortui* [(65); PDB entry 1JJ2.pdb]. The 30S subunit of *Thermus thermophilus* [(66); PDB entry 1J5E] is shown for orientation. Proteins L4 (forest) and L22 (blue) are shown as spacefilling, as are 16S rRNA residues in the decoding center A1492 and A1493 (red) and 23S residues in the peptidyltransferase center (PTC; red) G2061, G2447 and A2451. 16S, 23S and 5S rRNA are shown as backbone. Figure rendered with PyMol (67).

Several other reports also suggest that alterations to components of the 50S subunit affect not only the 50S function, but can also have an impact upon the function of the small subunit. Previous studies from this laboratory have shown that a variety of 23S rRNA mutations including those at, or close to the peptidyltransferase center can affect decoding on the 30S subunit (10,12). Mutants of ribosomal protein L6 confer resistance to the misreading-inducing drug, gentamicin and have a ribosomal ambiguity (*ram*) phenotype (9). Moreover, genetic analyses indicate that the erythromycin resistance phenotype associated with an altered L4 protein can be modified by mutations in the small subunit ribosomal proteins (24,25). The accuracy of translation is also affected by several antibiotics including aminoglycosides and the 50S-targeted antibiotics chloramphenicol, erythromycin, tylosin and the oxazolidinones that stimulate stop codon readthrough and frameshift errors during decoding (26–28).

In this report, we describe the effect of alterations in ribosomal proteins L4 and L22 on the accuracy of decoding. Our results indicate that while the L22 mutant is unaffected in decoding, the slow-growing L4 mutant affects readthrough of stop codons at the ribosomal A site, stimulates initiation from non-AUG codons at the ribosomal P site and affects the utilization of mutant initiator tRNAs and interactions with a range of antibiotics. These data indicate that L4 mutant ribosomes are compromised in multiple steps of protein synthesis and suggest that the interplay between ribosomal subunits modulates the ribosome's interactions with tRNAs, translation factors and antibiotics.

MATERIALS AND METHODS

Bacterial strains and plasmids

The erythromycin resistant mutants N281 (*rplV281*) and N282 (*rplD282*) carrying altered forms of ribosomal proteins L22 and L4, respectively, (20,21) were obtained from Dr Scott Champney (East Tennessee State University, Johnson City, TN). A set of isogenic Lac$^-$ strains carrying these mutations was constructed by transducing MC169 [F^- Δ(*lac-pro*) *thi*$^-$ *gal*$^-$ *cysG*::Tn5] to cysteine independence and erythromycin resistance with phage P1 grown on N282 or N281 to give strains MC251 and MC301, respectively. An isogenic, erythromycin-sensitive transductant (MC252, *rplD*$^+$, *rplV*$^+$) was also recovered and used as the wild type control strain for these experiments.

All of the *lacZ* plasmids used in this study have been described previously. In the frameshift and stop codon constructs, frameshifting or stop codon readthrough at target sequences in the 5′ end of the *lacZ* coding region is required in order to synthesize full-length β-galactosidase. Plasmids p12-6, p34-11 and p415 carry stop codons in the 5′ region of the *lacZ* mRNA (29). Plasmid pdnaX-FSs contains the signal directing frameshifting in the *dnaX* gene but lacks both stimulatory elements (30). Plasmid pL2HIVW carries the HIV-1-derived frameshift signal and stem–loop stimulatory element (31). Plasmids pLM211 and pLM44 contain short frameshifting windows constructed to examine +1 frameshifting at CCN proline codons (32). A variant of the frameshifting signal from the *prfB* mRNA encoding release factor 2 (RF2) is present in pRF-CUG (33). In plasmids pSG413 and pSG415, the AUG of the wild-type *lacZ* sequence has been replaced with CUG and AUC, respectively (29). Plasmid pGLZ22 contains a T4 gene 60–*lacZ* fusion and pGLZ23 is a derivative carrying a precise deletion of the 50 nt region bypassed during translation of gene 60 mRNA (34). Plasmid pDB wt is a fusion between the lambda c*I* repressor and *lacZ* (35). Transcription from the P_{RM} promoter in this construct produces a leaderless c*I*-*lacZ* mRNA. The wild-type *lacZ* gene is carried on plasmid pAC90.91 (36). In plasmid pSG500, the AUG initiation codon is replaced with a UAG codon. Initiation at UAG requires the

presence of a *metY*-derived, UAG-decoding initiator tRNA (37). Plasmids expressing initiator tRNAs were derived from pRSVCATam1.2.5 (38,39).

Codon 70 of the β-lactamase gene on the pTZ18u-derived (USB Corporation) kanamycin and trimethoprim resistant plasmid pKTA1, was mutagenized using the Quickchange kit (Strategene). The wild-type serine codon was changed to a GGC glycine codon (pKTS70G), a UUC phenylalanine codon (pKTS70F) or a UGG tryptophan codon (pKTS70W).

β-Galactosidase and β-lactamase assays and MIC determinations

Cells to be assayed for β-galactosidase activity were grown in minimal E medium (40) containing glucose (0.2%) thiamine, proline and casamino acids together with any necessary antibiotics. β-Galactosidase was assayed from logarithmically growing cells as described previously (29). β-lactamase activity in sonicated extracts was assayed as described using Nitrocefin (Oxoid, UK) as a substrate (41). Minimal inhibitory concentrations (MICs) were determined using a 2-fold dilution series of each antibiotic in 96 well microtiter dishes (42).

Table 1. Effects of *rplD* and *rplV* mutations on decoding fidelity

	Wild type	*rplD282* (L4)	*rplV281* (L22)
lacZ *mutants*[a]			
Stop codon readthrough			
p12-6 (UAG)	145 ± 11	354 ± 28	156 ± 9
p34-11 (UGA)	255 ± 8	424 ± 25	287 ± 13
p415 (UGA)	402 ± 34	750 ± 49	465 ± 34
Frameshifting			
pdnaX (−1 fs)	225 ± 49	591 ± 109	236 ± 27
pL2HIV-W (−1fs)	109 ± 9	221 ± 17	93 ± 5
pLM211 (+1 fs)	217 ± 59	404 ± 95	209 ± 24
pLM44 (+1 fs)	74 ± 3	194 ± 41	69 ± 9
pRF-CUG (+1 fs)	256 ± 19	363 ± 46	220 ± 24
Non-AUG initiation			
pSG413-CUG	493 ± 20	916 ± 154	473 ± 63
pSG415-AUC	482 ± 47	910 ± 163	422 ± 12
Bypassing (T4 gene 60)			
pGLZ22	295 ± 19	219 ± 16	272 ± 26
pGLZ23	2493 ± 116	2651 ± 97	2172 ± 89
Wild-type *lacZ*			
pDB wt (leaderless)	758 ± 88	1222 ± 52	717 ± 32
pAC90.91 (leadered)	5186 ± 798	4986 ± 931	4689 ± 1351
β-*lactamase mutants*[b]			
Missense suppression			
Ser70UUC	1.0 ± 0.5	0.9 ± 0.1	1.0 ± 0.2
Ser70GGC	2.8 ± 0.3	4.5 ± 0.4	3.2 ± 0.8

Units of activity represent the mean (± SE) of assays of at least three independent cultures.
[a]β-galactosidase activity is given in Miller units (64).
[b]Units of β-lactamase activity are nanomoles of nitrocefin hydrolyzed per min per μg of protein (41).

RESULTS

Miscoding properties of erythromycin resistant ribosomes: readthrough of stop codons, frameshifting and bypassing

An isogenic set of Lac^{-} strains expressing either wild-type or mutant L4 or L22 proteins was constructed and each strain was transformed with a variety of plasmids carrying nonsense or frameshift mutations in the 5′ region of the *lacZ* gene. The results of β-galactosidase assays of these strains (Table 1) indicate that the L22 mutant does not differ from wild type in its ability to shift reading frames or read through stop codons. In contrast, the L4 mutant supports an ∼2-fold increase in the levels of readthrough of UAG and UGA stop codons and a similar increase in both +1 and −1 frameshifting in a variety of sequence contexts. Some of the previously reported mutations in, e.g. helix 69 of 23S rRNA promote similarly modest increases in frameshifting and readthrough (10). However, mutations in helix 89 of 23S rRNA, or growth in the presence of oxazolidinone antibiotics promote much higher levels (at least 7-fold increases) of readthrough and frameshifting (10,27) The frameshift constructs examined here involve ribosome slippage by one or two bases. Bypassing of extended sequences occurs during translation of the T4 gene 60 mRNA and requires elaborate recoding signals (43). Ribosomal protein L9 appears to participate in this bypassing event as L9 point mutants or a complete lack of L9 influences bypassing efficiency both in the T4 gene 60 mRNA and at shorter bypassing events (44). However, neither the L4 or L22 mutant proteins affect gene 60 bypassing in construct pGLZ22, relative to a control plasmid, pGLZ23, indicating that these mutations differ qualitatively from the L9 mutations.

Missense suppression

Stop codon readthrough involves decoding of a termination triplet by a near-cognate tRNA that competes with the release factor for binding to the ribosomal A site. Miscoding at sense codons involves competition between cognate and near-cognate tRNAs and can be measured by examining missense suppression at positions that are essential for catalytic activity in a reporter protein. In the β-lactamase enzyme, a serine or cysteine at position 70 is required for function (45,46). The GGC (glycine) and UUC (phenylalanine) codons can be misread as serine via first or second position mismatches, respectively, and UGG (tryptophan) can be misread as cysteine (2). The wild-type AGC serine 70 codon was changed to each of these three codons by site-directed mutagenesis, the mutant plasmids were introduced into wild type, *rplD282* and *rplV281* strains and the transformants were examined for growth on ampicillin-containing medium. The UGG mutant failed to support growth of any strain at any ampicillin concentration while the GGC and UUC mutants showed residual levels of ampicillin resistance in all strains. Measurements of β-lactamase activities supported by the Ser70GGC and Ser70UUC mutants in wild type and the L4 and L22 mutants showed that the UUC codon was not subject to misreading by either type of mutant ribosomes. However, the strain carrying the altered L4 protein supported small but reproducible (1.6-fold) increases in activity of the Ser70GGC mutant. The same β-lactamase mutant had previously been constructed by Toth *et al*. (46) and shown to be misread as serine by ribosomes carrying an altered S4 protein that promotes high levels of miscoding. Thus, in addition to promoting readthrough of stop codons, these data suggest that the mutant L4 protein also promotes miscoding of sense codons in the ribosomal A site.

Missense suppression and readthrough of a stop codons requires decoding by a near cognate tRNA and reflects events occurring at the A site. While the stability of the P site-bound tRNA has emerged as the prime determinant of frameshifting potential (47), switches in reading frame may occur by several other mechanisms. For instance, P site slippage can be influenced by near-cognate tRNAs binding to the A site that are then prone to slippage into alternate reading frames upon translocation to the P site (48). Consequently, while increases in missense suppression and stop codon readthrough provoked by mutant L4 reflect an altered A site function, frameshifting events can reflect perturbation of ribosomal A and/or P sites.

Miscoding at the ribosomal P site: initiation at non-AUG codons and on leaderless mRNAs

All elongator tRNAs access the P site by first binding to the A site where tRNAs are actively screened for correct codon–anticodon pairing by the ribosome (7). Only during the initiation phase of translation does a tRNA bind directly to the ribosomal P site. The accuracy of initiation is ensured in part by the activities of the initiation factor IF3 which discriminates against non-initiator tRNA, non-AUG codons and mRNAs lacking leader sequences (37). However, the structure of rRNA residues close to the ribosomal A and P sites also influence the accuracy of initiation and we have previously described 16S rRNA mutations that affect tRNA–mRNA interactions during both initiation and elongation (29). The data presented in Table 1 show that the L4 mutant ribosomes support 2-fold increases in initiation from AUC and CUG codons. In addition, initiation from a leaderless *lacZ* construct is increased 1.6-fold in the L4 mutant while initiation from a conventional leadered *lacZ* construct (pAC90.91) is unaltered.

Use of initiator tRNAs by mutant ribosomes

The initiator tRNA is distinguished from elongator tRNAs by the several structural features including the lack of a base pair at positions 1–72 and the presence of three consecutive G-Cpairs in the anticodon stem (38). The availability of a specialized initiator tRNA has allowed the genetic analysis of initiator tRNA function. In this system, the anticodon of the specialized tRNA is altered so that it now decodes UAG instead of AUG. Chloramphenicol acetyl transferase (CAT) or *lacZ* reporter gene constructs that carry a UAG initiation codon allow monitoring of the activity of the specialized initiator tRNA (38,39). Mutations in the three anticodon stem G-C pairs impair the activity of the initiator tRNA as these mutant tRNAs now lack essential initiator tRNA features and are discriminated against by IF3 (37,39). Using this system, we have asked if the L4 mutant that affects the use of non-AUG initiation codons also influences initiator tRNA selection. Previous analyses have shown that affinity of the formylated, specialized tRNA for the ribosome is one of the several factors limiting initiation on the CAT/*lacZ* reporter mRNAs (49). The data presented in Table 2 show that the L4 mutant ribosomes enhance slightly the activity of the intact, specialized tRNA (called U35 A36 in Table 2), suggesting that the L4 mutation may affect its affinity for the ribosomal P site. In addition, the mutant ribosomes compensate in part (1.4- to 2.5-fold increases in activity) for the defects in activity of the specialized tRNA caused by substitutions in the three G-C anticodon stem pairs. Thus, several aspects of the initiation process normally sensed by IF3 are altered in the L4 mutant. Together, these data suggest that ribosomes carrying the altered L4 protein are defective in tRNA–mRNA–ribosome interactions at both A and P sites during elongation and at the P site during the initiation of protein synthesis.

Table 2. Effect of mutant L4 on activity of initiator tRNAs

metY tRNA mutants	Wild-type ribosomes	*rplD282* (L4) ribosomes
U35 A3	5888 ± 216	7154 ± 460
C30 G40/U35 A36	1295 ± 236	3926 ± 203
C30 G40 U29 A41/U35 A36	318 ± 33	574 ± 101
C30 G40 U29 A41 A31 U39/U35 A36	240 ± 16	332 ± 37

Numbers represent units of β-galactosidase activity obtained in wild-type or L4 mutant strains carrying a *lacZ* plasmid with a UAG initiation codon [pSG500; (37)] and each of the indicated initiator tRNA mutants on pRSVCATam1.2.5-derived-plasmids (38). Cells were grown in minimal medium with required supplements, tetracycline (12.5 mg/l) and ampicillin (200 mg/l). Each β-galactosidase activity measurement represents the mean (± SE) of assays from at least three independent cultures.

Table 3. MICs of wild-type and erythromycin resistant strains

Antibiotic	Wild type MIC (μg/ml)	*rplD282* (L4)	*rplV281* (L22)
Kasugamycin	250	125	250
Paromomycin	7.8	3.9	3.9
Streptomycin	4.9	2.4	4.9
Spectinomycin	31.3	7.8	31.3
Tetracycline	2.4	2.4	2.4
Fusidic acid	250	500	250

Altered antibiotic resistance in L4 and L22 mutants

Given the effects of the L4 mutant on decoding, we have asked if interaction with any antibiotic inhibitors of protein synthesis are also affected by the altered L4 protein. Resistance to a range of antibiotics, affecting distinct steps of protein synthesis was assessed by determining MICs for wild type, L4 and L22 mutant strains. The data presented in Table 3 show that while sensitivity to tetracycline does not differ between strains, the L4 mutant displays a 2-fold increase in sensitivity to kasugamycin, paromomycin and streptomycin and a 4-fold increase in sensitivity to spectinomycin. Conversely, the same L4 mutation increases the resistance to fusidic acid by 2-fold. The L22 mutant shows a 2-fold increase in sensitivity to paromomycin only. Both paromomycin and streptomycin promote misreading of the genetic code, as does the altered L4 protein itself. Several of the ribosomal mutants that have previously been shown to promote decoding errors are hypersensitive to these aminoglycosides and consequently, it is not unexpected to observe an increased sensitivity to paromomycin and streptomycin in the L4 mutant (50,51). More surprising are the altered responses of L4 mutant ribosomes to kasugamycin which affects initiation as well as decoding (52), spectinomycin which affects translocation and fusidic acid which affects ribosome–EF–G interactions. Together these data suggest that in addition to the previously described defect in peptide bond formation (20), L4 mutant ribosomes

are affected in their interaction with several antibiotics as well as multiple steps of translation including initiation, decoding and reading frame maintenance.

DISCUSSION

The analysis of ribosomal function by characterization of ribosomal mutants and the use of ribosome-targeted antibiotics has provided valuable insights into the mechanism of protein synthesis and the structure of the translational machinery. The most easily-interpreted mutants and inhibitors are those that affect single, discrete step(s) of translation without influencing other aspects of the process. However, because of the interconnectedness of the various stages of protein synthesis, many mutants and antibiotics affect multiple steps of translation. Thus, while certain ribosomal protein S12 mutants confer resistance to streptomycin and restrict A site miscoding events, the same mutants also increase the affinity of peptidyl tRNAs for the ribosomal P site (53) while other S12 mutants affect the spontaneous translocation of tRNAs (54). Similarly, aminoglycoside antibiotics have a well-described effect on miscoding, but have also been reported to affect the initiation and translocation steps (55,56). The ribosomal protein L4 mutant described here was originally isolated as an erythromycin resistant mutant and was subsequently shown to decrease the rate of peptide bond formation (20). In this report, we show that decoding and antibiotic interactions on the 30S subunit are also influenced by the altered conformation of the 50S subunit found in the L4 mutant and we conclude that the L4 alteration has numerous effects on the structure and function of both 50S and 30S subunits.

While mutations in L4 and L22 each confer high level resistance to erythromycin, only the L4 mutation affects the fidelity of translation. This distinction is consistent with the biochemical analyses which showed that the mutant ribosomal proteins affected erythromycin resistance by different mechanisms and that only the L4 mutant affected peptide bond formation (20). Moreover, structural analyses of the L4 and L22 erythromycin resistant ribosomes showed that both 50S and 30S subunits from each mutant differed from one another and from the wild-type structure (23). Thus, while the two mutations confer erythromycin resistance, they differ in the mechanism of resistance and have differential effects on ribosome structure, decoding and peptide bond formation. Among the regions of the 50S subunit altered in the L4 mutant were helix 69 (h69) of 23S rRNA and the L7/L12 stalk. Mutations in both of these regions have previously been shown to affect the accuracy of decoding (8,10). Helix 69 forms part of bridge B2a connecting the decoding center and the 50S subunit (16) and an altered h69 conformation thus has the potential to affect the function of the decoding center directly. The L7/L12 stalk forms part of the binding site for translation factors and may affect decoding by altering interaction with initiation, elongation and termination factors. Earlier genetic studies showed that the erythromycin resistance associated with the altered L4 protein could be reversed by a combination of mutations in 30S proteins, S12 and S5 (24,25). Together, these genetic and structural data indicate that alterations in the structure of one subunit influence not only that subunit's function, but may also affect the conformation and function of the other subunit.

The peptidyltransferase center of the 50S subunit is responsible for both peptide bond formation during elongation and peptidyl-tRNA hydrolysis at the termination phase of translation. Mutations in 23S rRNA residues at, or close to the active site of the 50S subunit have been shown to have distinct effects on both of these activities (57). In addition, the termination reaction is sensitive to antibiotic inhibitors of peptidyltransferase activity (58). Mutant 50S subunit components may thus influence stop codon readthrough and the frameshifting events occurring at stop codons (10,33) through effects on the termination reaction and this likely underlies some of the miscoding properties of rRNA mutants in the catalytic site (12) as well as the influence of some 50S inhibitors on stop codon readthrough (27,28). Although it is unlikely, we cannot exclude the possibility that slow peptide bond formation also affects missense suppression. However, the spectrum of miscoding errors exhibited by the L4 mutant is not limited to termination-related events but extends to initiation events. This consideration, together with the altered response to 30S antibiotics argues that the function of several ligand binding sites of the 30S subunit is altered by 50S subunits carrying the mutant L4 protein.

The altered residue (Lys 63) in the mutant L4 is some 73 Å from the decoding center and 28 Å from the catalytic center (Figure 1) (16) and so any effects of L4 on decoding and peptide bond formation must be indirect. Moreover, modulation of the decoding functions of the 30S subunit by an altered 50S subunit requires that the structural changes caused by the mutant L4 be propagated across the inter-subunit space to the decoding center. In one model to explain the influence of mutant L4 on ribosome function, the effect is primarily on the tRNA which spans both subunits. In this model, the mutant L4 alters the placement of tRNAs on both subunits, leading to alterations in both peptidyltransferase and decoding activities. During translation, the two subunits are connected by tRNAs and by a series of RNA–RNA, RNA–protein and protein–protein bridges (16,59). In a second model, the changes elaborated by the mutant L4 on the large subunit are transmitted to the small subunit via altered inter-subunit connections. Consistent with the latter interpretation are the cryo-EM studies of empty, tRNA-free 70S ribosomes from the L4 mutant showing that the structures of both the 50S and 30S subunits are altered (23). Moreover, the altered response of the L4 mutant to antibiotics indicates that other ligand binding sites on the 30S subunit in addition to tRNA binding sites are affected by the alteration of L4 in the 50S subunit.

Many of the features of initiation that are altered in the L4 mutant are normally under the control of initiation factor IF3. Although the mechanism of action of IF3 still remains controversial, both hydroxyl radical footprinting and cryo-EM analyses suggest that the factor is distant from the P site tRNA and inspects codon–anticodon pairing indirectly by affecting the conformation of the ribosomal P site (60–62). The L4 mutation could thus affect initiation by altering the conformation of the 30S P site upon subunit joining, or conceivably, by influencing the ejection of IF3 from 70S initiation complexes. An effect of an altered large subunit on selection of initiation codons has also been observed with mutants of yeast ribosomal protein L16 (63). Interestingly, yeast L16 is

equivalent to *E.coli* protein L5 that forms part of inter-subunit bridge B1b and thus also has the potential to influence the structure of the small ribosomal subunit directly (16).

The sequential activities of decoding, peptide bond formation and translocation require that the functions of the two subunits be regulated and one function of the inter-subunit bridges may be to transmit signals between subunits. The data presented here indicate that modest alterations to a 50S subunit protein can have large effects on the structure and function of both subunits and are consistent with the proposal that inter-subunit communication plays a central role in the coordination and regulation of the activities of large and small subunits during translation.

ACKNOWLEDGEMENTS

We are indebted to W.-C. Mimi Lee for her input during the early stages of this project. We thank Jill Thompson for comments on the manuscript and George Q. Pennabble for his indefatigable insights. This work was supported by grant GM19756 from the US National Institutes of Health to A.E.D.

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