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Real-Time Evidence for EF-G-Induced Dynamics of Helix 44 in 16S rRNA

Douglas R. Tanner, Emily G. Hedrick, and Walter E. Hill*

Division of Biological Sciences and Center for Biomolecular Structure and Dynamics, The University of Montana, Missoula, MT 59812, USA

Abstract

The penultimate stem-loop of 16S ribosomal RNA (rRNA), helix 44, plays a central role in ribosome function. Using time-resolved dimethyl sulfate (DMS) probing, we have analyzed time-dependent modifications that occur at specific bases in this helix near the decoding region, resulting from the binding of elongation factor G (EF-G) in various forms. When EF-G-GTP is bound to 70S ribosomes, bases A1492 and A1493 are immediately protected, while other bases in the region show either no change or enhanced modification. When apo-EF-G is bound to 70S ribosomes and GTP is added, substantial transient time-dependent enhancement occurs at bases A1492 and A1493, with somewhat less enhancement occurring at base A1483, all in the first 45 ms. When mRNA and deacylated tRNAs are bound to the 70S ribosome and EF-G-GTP is added, bases A1492 and A1493 again show substantial and continued enhancement, while bases A1408, A1413, and A1418 all show time-dependent protection. These results provide primary, real-time evidence that EF-G induces direct or indirect structural changes in this region as EF-G is bound and as GTP is hydrolyzed.

Keywords

dynamics; 16S rRNA; helix 44; EF-G; translocation

Introduction

Translocation is the process whereby two tRNAs bound to their respective mRNA codons are synchronously moved one codon relative to the ribosome. Since the tRNAs are primarily bound to the 50S ribosomal subunit, and the mRNA is primarily bound to the 30S ribosomal subunit, this transition necessitates multiple, exquisitely timed and choreographed transitional interactions between the ribosomal subunits, tRNAs, and mRNA involved. The primary director of this activity is the translational factor, elongation factor G (EF-G).

With the use of numerous physical techniques, it has been clearly shown that the ribosome itself, and the ribosomal RNA (rRNA), the tRNAs, and EF-G all go through numerous physical rearrangements to allow the codon to move forward the prescribed distance, carrying cognate tRNAs along, while the ribosome resets itself in anticipation of the next tRNA. Details of these conformational changes have been identified using X-ray

crystallography,^{1–7} cryo-electron microscopy,^{8–12} single-molecule Förster resonance energy transfer,^{13–20} and molecular dynamics simulation^{21,22} techniques. Cryo-electron microscopy provides a valuable and proven method to delineate intermediate structures in the translocation process at less than 10 Å resolution. Single-molecule Förster resonance energy transfer can identify the gross dynamic changes occurring as translocation takes place. These latter two approaches can be rationalized in great detail using molecular dynamics simulation approaches to model the changes occurring. All of these techniques are imbedded in the foundation of X-ray crystallography, which provides static, atomic-resolution structures at various stages of translocation (see Ref. 23 for an excellent review). From these studies, amazing details of the translocational process have begun to emerge. Major conformational changes of the ribosome, including intersubunit rotation, small-subunit head swiveling, domain closure, locking and unlocking of tRNA movement, and evidence for a Brownian pawl, have emerged (see Refs. 23–27 for reviews). Details of such conformational changes will be elicited by observing the dynamics of single residues in millisecond time domains, as reported in this study.

The interaction of EF-G with the ribosome is central to the entire translocation process and involves not only structural changes of EF-G itself as it is bound to the ribosome and when GTP is hydrolyzed^{28–30} but also structural changes of the ribosome as well, both before and after GTP is hydrolyzed.^{13,16,31–34} The timing of these structural changes is critical. Munro *et al.* have reported that translocation is a three-step process, each step consuming about 25 ms;¹⁶ these times are substantially in agreement with the results obtained earlier by Wen *et al.*, who used optical tweezers to show that the entire translocation step itself occurred in ~100 ms.³⁵

However, even with the application of these powerful techniques, the details of the dynamics induced by EF-G on the ribosome are still unclear. Time-dependent structural changes of the ribosome have been reported^{8,33,36} but lack sufficient resolution to identify specific rRNA nucleotides undergoing induced changes. However, by using time-dependent dimethyl sulfate (DMS) modification,^{37–39} we are able to analyze the changes in chemical modification occurring at specific nucleotides down to 10 ms as EF-G is bound to the ribosome and GTP is hydrolyzed.

We especially looked for changes occurring in the first 100 ms, since, as noted above, translocation has been reported during this time frame.^{16,35} Our results provide evidence that after GTP hydrolysis by EF-G during translocation, domain IV of EF-G apparently undergoes a conformational change that places it in close proximity to the proximal end of helix 44 found in the decoding region of the ribosome.^{30,40,41} We have limited this study to nucleotides in the proximal end of helix 44 of 16S rRNA in this region and have identified, to the extent possible, causes for the modification changes observed, using extant crystal structures (Fig. 1). To our knowledge, this is the first evidence showing real-time structural changes occurring with individual nucleotides as EF-G is bound to the ribosome and GTP is hydrolyzed.

Results

Three experimental approaches were used to identify time-dependent changes in chemical modification of individual nucleotide bases in helix 44 as EF-G was bound to 70S ribosomes. The first approach was to bind EF-G-GTP to 70S ribosomes for short delay times in a quench-flow apparatus, following which the mix was instantly modified with DMS and quenched, and the collected sample was analyzed by primer extension.^{37,39,42} A second approach was to bind apo-EF-G to 70S ribosomes initially, following which we added GTP from the second syringe, again monitoring the time-dependent changes in modification

taking place with those bases close to the decoding region. The purpose of this experiment was to monitor changes in modification of 16S rRNA when GTP was added and hydrolyzed. In a final approach, we added EF-G-GTP to a mix consisting of 70S ribosomes, mRNA, tRNA^{fMet}, and tRNA^{Phe} and monitored time-dependent changes that occurred as EF-G-GTP was bound. As control experiments, we also looked at modification changes occurring as apo-EF-G, EF-G-GDP, and EF-G-GDPNP (the non-hydrolyzable form of GTP) each were added to 70S ribosomes or to 70S ribosomes, mRNA, and two tRNAs. A gel showing time-dependent modifications at base A1483 as EF-G-GTP and EF-G-GDP were added is shown in Fig. 2.

We found dramatic time-dependent modification changes in six bases, A1492, A1493, A1483, A1418, A1413, and A1408, all located in the proximal one-third of helix 44 of 16S rRNA near the decoding region (Fig. 1). Although some less definitive changes were observed at other bases, these six bases all showed at least 50% protection or enhancement under one or more of the experimental conditions used, well above the statistical intensity fluctuations inherent in the gels of primer extension analyses. A compilation of the results of the first and third approach are found in Table 1 and the results from adding GTP to apo-EF-G bound to 70S ribosomes are found in Table 2.

Base A1408

Located on helix 44 of 16S rRNA on the opposite side of the internal bulge containing bases A1492 and A1493 on the other strand, A1408 is the 16S rRNA portion of bridge B2a, the other portion of this bridge being bases A1913–A1914 of helix 69 of 23S rRNA (Fig. 1b).⁴³ When EF-G-GTP is added to 70S ribosomes, this base shows significant protection (Table 1; Fig. 3). Although these results suggest immediate time-dependent occlusion of the solvent environment as EF-G-GTP is added, no change occurs when either EF-G-GDP or EF-G-GDPNP was added.

We then asked whether the protection seen was due to the binding of EF-G or caused by GTP hydrolysis. When we bound apo-EF-G to 70S ribosomes and then added GTP to the mix, a different pattern emerged (Table 2; Fig. 4). In this case, as we compared the relative intensities to the values obtained for 70S–EF-G as the control, we found initial enhancement (<15 ms) in the modification of A1408, which gradually became attenuated after 30 ms until it became ~20% protected at equilibrium (Table 2). The amount of enhancement is less than that of bases A1492 and A1493 under these conditions, but the pattern is similar, occurring in the first 30 ms.

As we added EF-G-GTP to the mix containing 70S ribosomes, mRNA, and the two deacylated tRNAs, about 15% protection occurred, increasing to ~35% in time, even though enhancement was observed with bases A1492 and A1493 at these same times (Table 1; Fig. 5), but when EF-G-GDPNP was added to the mix, a small transient enhancement occurred, moving to a 25% protection at equilibrium (Table 1).

Base A1413

A1413 is located more distantly from the decoding region, but on the same strand as A1408, yet on the opposite side of helix 44. A1413 is in direct contact with base A909 of helix 27 of 16S rRNA (Fig. 1c), which, in turn, is in direct contact with Lys17 (Lys21 in *Thermus thermophilus*) in the N-terminal arm of protein S12. The body of S12 is in direct van der Waals contact with EF-G.¹

As EF-G-GTP is added to 70S ribosomes, a substantial protection occurred at base A1413 in less than 30 ms, resulting in ~45% protection at equilibrium (Table 1; Fig. 3). When EF-G is bound in the GDP form, a slight enhancement occurs, but there is no change when EF-G-

GDPNP (Table 1) or apo-EF-G is bound (data not shown). When apo-EF-G is bound to 70S ribosomes, and GTP is added, slight protection occurs (Table 2; Fig. 4). However, when the tRNAs and mRNA are present with 70S ribosomes, substantial (~45%) protection at base A1413 occurs immediately (<15 ms) upon the addition of EF-G-GTP, followed by a continual increase in protection to ~60% at equilibrium (Table 1; Fig. 5). There is no change in modification when EF-G-GDPNP is added to the 70S ribosomes, mRNA, and tRNA mix (Table 1).

Bases A1418 and A1483

These bases are adjacent to each other in helix 44, base A1418 being the 16S portion of bridge B5, coupled to portions of helix 64 of 23 S rRNA, whereas base A1483 is part of the 16S portion of bridge B3⁴³ (Fig. 1d). As EF-G-GTP is added to 70S ribosomes, both A1418 and A1483 show significant time-dependent enhancement, as shown in Table 1 and Fig. 3. Similar enhancement occurs with EF-G-GDP, but not with EF-G-GDPNP (Table 1). There is no change in modification with apo-EF-G bound with GDPNP added (data not shown).

A major difference in the modification pattern of these two bases occurs when apo-EF-G is bound initially to the 70S ribosomes and GTP is added, in which case a slight protection occurs at base A1418, but significant transient enhancement (~50%) occurs with base A1483 (Table 2; Fig. 4). When GDPNP is added to apo-EF-G bound to 70S ribosomes, there is no change in modification at either base from that seen with the apo-EF-G-70S control (data not shown).

When EF-G-GTP is added to the 70S ribosomes with mRNA and tRNAs present (Table 1; Fig. 5), a slight enhancement in modification occurs. However, addition of EF-G-GDPNP to 70S ribosomes, mRNA, and tRNAs apparently reverses this, causing enhancement at base A1418 and protection at base A1483, except at equilibrium.

Bases A1492–A1493

Perhaps the most studied bases in the decoding region, A1492 and A1493, were first identified by Puglisi's group as essential for discrimination of codon–anticodon base pairs⁴⁴ and later confirmed by Ramakrishnan's group with crystallographic evidence (Fig. 1a).⁴ In the crystallographic study, they were found to flip out of helix 44 into the A-minor groove created by the codon–anticodon interaction between the A-site tRNA and mRNA.⁴ These bases have been shown to play a central role in stabilizing the mRNA–tRNA interaction between cognate tRNAs and their codons.² A major impetus for this study was to see if we could “watch” these bases move from a protected to a nonprotected state in real time.

Initially, as we added EF-G-GTP to 70S ribosomes, we observed immediate (<30 ms) and substantial (~50%) protection at both bases A1492 and A1493 as shown in Table 1 and Fig. 3. Such protection did not occur when apo-EF-G (data not shown), EF-G-GDP, or EF-G-GDPNP was added to the 70S ribosomes.

When we bound apo-EF-G initially to the 70S ribosomes and then added GTP, we were surprised to find, using the 70S+ EF-G value as a control, that both A1492 and A1493 were enhanced about 100% by 30 ms, falling to about half that amount at equilibrium (Table 2; Fig. 4). We had expected that the addition of GTP to pre-bound EF-G on 70S ribosomes would give the ~50% protections observed when EF-G-GTP was added to 70S ribosomes, but instead a pronounced transient enhancement occurred.

When mRNA, tRNA^{Fmet}, and tRNA^{Phe} were present on the 70S ribosomes, and EF-G-GTP was added, a similar pattern of transient enhancement occurred, except in this case, A1492 and A1493 remained exposed at equilibrium (Table 1; Fig. 5). These results indicate a

dramatic increase in solvent availability of the N₁ atoms of bases A1492 and A1493 as EF-G-GTP is added, when mRNA and tRNAs are present. This enhancement did not occur when EF-G-GDPNP was added to the 70S, mRNA, and tRNAs mix, except at equilibrium (Table 1).

Base A702

This base is neither in helix 44 nor near the decoding region, but is on the platform of the 30S subunit. However, it has been implicated in intersubunit rotation, in which in the rotated form it moves away from helix 68 of 23S rRNA by 13 Å, making the N₁ of A702 available for DMS probing.^{5,6,3045,46} Very recent studies by Noller's group looking at the crystal structure of RF3 trapped in the GTP state on a rotated conformation of the ribosome show that although A702 is moved 13 Å, rearranging bridge B7a, A702 becomes hydrogen bonded to phosphate 1847 of 23 S rRNA in the rotated form, which may occlude solvent access to A702.

In any case, we have analyzed the modification of this base under our conditions and have found that at equilibrium, binding apo-EF-G to 70S ribosomes gives ~35% enhancement, but binding EF-G-GTP, EF-G-GDP, or EF-G-GDPNP gives no statistical change (data not shown). When we analyzed the time-course studies, we had expected to see some transient exposure and then protection, but our results all produced no more than statistical change in modification at this base.

Discussion

The results of this study provide real-time definition to structural changes occurring under the conditions of the experiments performed. Such changes are induced either by the binding of EF-G itself or by structural changes induced in EF-G by binding to the ribosome or by GTP hydrolysis. The interaction of EF-G with the ribosome then causes structural changes to occur in discrete portions of the rRNA (and likely the proteins as well), the cumulative effect being more global conformational changes in the ribosomal subunits and ribosome itself. We have tried to identify the causes of the changes in modification we observed at the six bases studied.

Base A1408

Overall, we conclude that most of the time-dependent changes in modification of base A1408 result from structural changes induced on the 70S ribosome by EF-GTP as GTP is hydrolyzed, since there is no marked activity at this base with either EF-G-GDP or EF-G-GDPNP added to 70S ribosomes. This is true whether mRNA and tRNAs are present or not. It is possible that since Arg499 of domain IV of EF-G is within 4 Å and partially stacks over base A1408 when EF-G is in the posttranslocational state,¹ this may cause the protection observed at A1408 as EF-G-GTP is bound to the 70S ribosomes and domain IV moves due to GTP hydrolysis.⁴⁰

With tRNAs and mRNA present, there is possibly an additional reason for the time-dependent protection observed at this base. Helix 69 of 23S rRNA is in direct van der Waals contact with both EF-G domain IV and P-site tRNA in the posttranslocational state.¹ Thus, these contacts and the resulting compression of helix 69,⁴⁵ which is in van der Waals contact with A1408 (through bridge B2a), may induce the protection seen at A1408 as domain IV of EF-G moves due to GTP hydrolysis. The lack of significant time-dependent variation of modification of A1408 as EF-G-GDPNP is bound to the 70S ribosome, mRNA, and tRNAs mix provides further evidence that the protection observed for base A1408 is caused by movement of domain IV of EF-G. An additional argument can be made for a mechanical

deformation or stabilization of helix 44 due to the direct contact of domain IV of EF-G with G1494.

Although we cannot separate the effects of each of these contacts, together they could lead to the slight enhancements we observed as GTP is hydrolyzed, or in the increased protection of A1408, even though the counterpart bases on the other strand, A1492 and A1493, experience significant enhancement in modification during the same time frame (see below and Table 1 and Fig. 5).

Base A1413

To try to find a possible reason for the changes observed in this base, we note that although base A1413 is not proximal to EF-G itself, it does make van der Waals contact with base A909 of helix 27 of 16S rRNA. Base A909 is in direct van der Waals contact with the N-terminal arm of protein S12 (Lys17 in *Escherichia coli* or Lys21 in *T. thermophilus*). The adjacent nucleotide, C1412, contacts Lys57 of protein S12 during domain closure of *T. thermophilus* ribosomes,³ but we see no change in modification of this base under our conditions. This may be due to differences between *T. thermophilus* and *E. coli* ribosomes. Thus, the modification results on A1413 may be caused by protein S12 indirectly interacting with base A1413.

Protein S12 has long been known to play a central role in translation, being an active player in streptomycin sensitivity.^{47,48} Recent mutational analyses in *E. coli* have confirmed that phenotypes showing hyper-accurate decoding have mutations near contact points with rRNA found in the decoding region.^{49,50} Specifically, the interaction of protein S12 with helix 27 is shown to occur when cognate tRNA is present.⁴ Our results corroborate these findings.

It appears that when EF-G-GTP is bound to the 70S ribosome, with or without tRNAs and mRNA, protein S12 is contacted and likely moved, relaying that movement through Lys17 in its N-terminal arm through A909 of helix 27 to A1413. Recently, we reported that such motion of the N-terminal arm of protein S12 is likely responsible for transient modification changes in the more distant helix 11 loop of 16S rRNA.⁵¹ Such movement of the N-terminal arm of S12 may well cause base A909 to make initial or increased contact with base A1413 and that N₁ of base A1413 may be pushed into contact with N₁ of G1487, causing the time-dependent protections observed.

However, the total lack of protection or enhancement when EF-G-GDP or EF-G-GDPNP is added either to 70S ribosomes or to the mix of 70S ribosomes with mRNA and tRNAs suggests that the protections observed are caused by structural changes occurring in EF-G as GTP is hydrolyzed. The results from the 70S ribosomes with apo-EF-G bound when GTP is added show protection as well, but it is not as pronounced. Analysis of the crystal structure of the posttranslocational EF-G on the ribosome shows that domain III of EF-G is contacting the globular portion of S12. These results, taken together, suggest a GTP-induced structural change in EF-G occurring not only in domain IV but also in other domains of EF-G.^{22,28} Such structural changes in other domains of EF-G have been found in the crystal structure of RF3 and the ribosome.³⁰

Hence, although we see protection of A1413 that mimic those seen with A1408, it appears that those occurring with base A1413 are transmitted through protein S12 and are due to structural changes occurring in EF-G, but may not be directly attributable to domain IV movement.

Bases A1418 and A1483

From crystallographic results of *E. coli* 70S ribosomes, N₁ of A1418 is hydrogen bonded with G1948 of 23S rRNA (bridge B5), and N₁ of A1483 is hydrogen bonded to G1959 (bridge B3).^{6,43} Our results suggest that when either EF-G-GTP or EF-G-GDP (but not EF-G-GDPNP) is added to 70S ribosomes, these hydrogen bonds are broken, allowing DMS to methylate the N₁ atoms of both bases A1418 and A1483 (Table 1).

But what might cause the disruption of these hydrogen bonds? The binding site of EF-G in the posttranslocational state is distant from either of these bases, yet both of them are exposed in a time-dependent manner as EF-G-GTP or EF-G GDP is bound to 70S ribosomes. With EF-G-GDPNP, no such enhancement is observed, suggesting that GTP hydrolysis and movement of domain IV of EF-G is not directly responsible for the disruption of these hydrogen bonds. Hence, the cause of this disruption cannot be ascribed directly to contact with EF-G or GTP hydrolysis. We posit that it is likely due to torsional pressures due to the deformation of helix 44 as EF-G is bound and intersubunit rotation and/or head swiveling occurs. The fact that EF-G-GDP causes these modification changes and EF-G-GDPNP does not suggests that the structure of ribosome-bound EF-G-GDP is different from that of ribosome-bound EF-G-GDPNP.

It has been posited by Cate's group that these bases may serve as the pivot point or point of rotation as the two subunits rotate relative to each other.^{6,45} Our results corroborate this. These bases, A1418 and A1483, occur at about two-thirds the distance (90 Å) up helix 44 from the distal end, about where bending of helix 44 is reported to occur.¹² As noted above, the time-dependent changes in modifications we observe at these bases may be a direct result of the torsional stresses on helix 44 caused by movement of the proximal end of helix 44 as ratcheting and/or head swiveling occur.^{6,11,45}

These bases also play an important role in ribosomal subunit association. We found in an earlier study that A1418 was among the first nucleotides to be protected from DMS modification in a time-dependent manner when the 50S and 30S ribosomal subunits were associated.³⁸ More recently, Remme's group, using an RNA modification interference approach, showed that both A1418 and A1483 were essential for subunit association.⁵²

Bases A1492 and A1493

The results we obtained for bases A1492 and A1493 were both exciting and surprising. Analysis of the crystal structure of the ribosome, when EF-G is bound in the posttranslocational GDP form, shows that although Met580 of EF-G domain IV actually makes a van der Waals contact with base A1493, the N₁ atoms of both A1492 and A1493 are not occluded by domain IV of EF-G directly. The initial protections we observed when EF-G-GTP was added to the 70S ribosomes must be due to an induced structural deformation of these bases into a protected site or a van der Waals contact of these bases by other moieties moved as EF-GTP is bound to the ribosome. Since there are no crystal results showing EF-G-GTP bound to the ribosomes, we cannot speculate as to why this initial protection on 70S ribosomes occurs, but it is very reproducible and very pronounced.

However, the results obtained when GTP is added to the apo-EF-G–70S complex provide strong evidence that the structural changes in EF-G that occur as GTP is added and hydrolyzed move bases A1492 and A1493 (or other intermediate moieties) in such a way as to cause A1492 and A1493 to become increasingly exposed to DMS modification within 30 ms, the time of which is quite similar to values identified with the translocation process itself.^{16,35} GTP hydrolysis is much more rapid than 45 ms;³⁶ thus, we are really observing the rRNA structural deformation caused by changes in EF-G structure as a result of GTP hydrolysis.

The fact that protection does not occur when apo-EF-G is bound and GTP is added or when mRNA and tRNAs are present is more readily reconciled with existing information. It is well accepted that GTP hydrolysis causes a structural change in EF-G, in which domain IV moves significantly.^{12,40} Such movement of domain IV of EF-G could readily cause the results we observe, causing bases A1492 and A1493 to become more solvent accessible. This is likely a direct result of these bases flipping out of the A-minor groove in the mRNA/A-site tRNA codon/anticodon interaction, where they were sequestered before EF-G-GTP was hydrolyzed. This would corroborate the results reported by the Ramakrishnan group⁴ and the results of Yusupov's group, showing the tip of domain IV of EF-G intimately involved in the A-minor groove.² In addition, our results show that this structural change in EF-G occurs within the first 30–45 ms after GTP is added to the apo-EF-G bound to the 70S ribosomes.

Base A702

Since we are very familiar with the many reports of the exposure of A702 as the ribosome is rotated,^{5,6,30,45,46} we were perplexed when we failed to see significant signal from this base in our time-course studies. Although we did see a significant enhancement at equilibrium when apo-EF-G was bound to the 70S ribosome, we found no modification change when mRNA and tRNAs were present. Crystal structures clearly show that A702 is displaced by 13 Å and bridge B7a is disrupted when intersubunit rotation occurs, and in the process, the N₁ of A702 should become solvent accessible.^{5,6,30,45,46} Indeed, one of the significant features obtained from the early studies of Moazed and Noller for the hybrid state was the exposure of A702 to DMS.⁵³ However, these studies were made under bench-top equilibrium conditions with DMS exposure being for extended times at 0 °C.

In light of the very recent results from Noller's group in which the structure of the ribosome with RF3 trapped in the GTP state shows additional hydrogen bonding of A702 with phosphate 1847 of 23S RNA in the rotated state,³⁰ it appears that even though there is significant movement of A702 as intersubunit rotation occurs, A702 may not remain exposed in the process. We had hoped to see even transient exposure, but after many efforts to identify such, we can only report that under our conditions and in the times we can observe such changes (down to 15 ms), no changes were apparent. Such negative results cannot be used to conclude that intersubunit rotation is not occurring, but rather that A702 may not really be accessible, except for very short periods of time.

General Results

As we distill the results found as various forms of EF-G are bound to the ribosome, some interesting insights appear. As EF-G-GTP is bound to the 70S ribosomes, substantive protection occurs at bases A1408, A1413, A1492, and A1493. At the same time, substantive enhancement occurs at bases A1418 and A1483. Although there is not yet a crystal structure of EF-G-GTP binding to 70S ribosomes, it is clear that binding EF-G-GTP to the vacant ribosome itself causes considerably structural change in the decoding region of helix 44. When mRNA and tRNAs are added, the protections remain at base A1413, but the protection at base A1408 occurs more slowly, suggesting a different mechanism for this protection when the mRNA and tRNAs are present. Surprisingly, bases A1492 and A1493 go from ~50–60% protection to ~30–50% enhancement when mRNA and tRNAs are present.

When EF-G-GDPNP is bound to the 70S ribosomes, there is very little, if any, change in the modification of any of the six bases. However, when mRNA and tRNAs are present, transient enhancement occurs at bases A1408 and A1418, while transient protection occurs at A1483, ultimately ending in enhancement at equilibrium. Taken together with the EF-G-

GTP results, it appears that GTP hydrolysis is responsible for the substantial changes occurring when EF-G-GTP is added, but the structure of EF-G-GDPNP seems sufficiently different to cause some transient modification behavior in some of the bases.

In turn, we see that EF-G-GDP is responsible for substantial enhancement of bases A1418 and A1483, as is EF-G-GTP, but these are the only two bases significantly affected by the binding of EF-G-GDP. EF-G in the GDP form normally dissociates from the ribosome following translocation, but to obtain crystal structures, the Ramachandran group used fusidic acid to stabilize the EF-G-GDP on the ribosome.¹ Under our conditions, EF-G-GDP causes enhancements of bases A1418 and A1483 out to the 30-min equilibrium time. However, as noted above, it appears that these two bases have no direct interaction with EF-G itself; hence, the enhancements observed may be a result of altered ribosome structure, even after EF-G-GDP has dissociated from the ribosome.

The EF-G-GDPNP equilibrium results were surprisingly high at several sites (see Table 1). It is expected that both EF-G-GTP after hydrolysis to EF-G-GDP will dissociate from the ribosomes, as will EF-G-GDP itself. However, EF-G-GDPNP will remain bound, even after 30 min. The result of this binding on the structure of the ribosome after that period of time is unknown, but our equilibrium results suggest that there may be structural changes that occur, resulting in increased solvent accessibility to these bases (see Table 1).

Since GTP causes domain IV of EF-G to move significantly, we can interpret changes that occur with EF-G-GTP and do not occur with either EF-G-GDPNP or with EF-G-GDP as those being caused by domain IV, or perhaps domain III movement within EF-G. We have attempted to summarize these results in cartoon form in the model proposed in Fig. 6.

Proposed Model

Analysis of the active nucleotides in helix 44 near the decoding region has provided, for the first time, insight into rRNA movement in the proximal end of helix 44 of the 30S subunit as head swiveling or intersubunit rotation (ratcheting) occurs.^{6,13,30,45,46} Other conformational changes, such as locking and unlocking,^{12,16} domain closure,^{3,25,54} and the Brownian pawl mechanism,^{23,24} will yet be assessed using time-dependent DMS probing. Time-dependent modification results at a given base may be due to conformational changes of the base itself, deformation of nearby structure, occlusion of the modified base by ligand proximity, or ionic perturbations of the solvent in the region. However, by using a mechanistic approach, we have identified possible causes of transient modification changes at most of the bases analyzed in this study. Future real-time analysis of translocation, looking at additional bases and using mutant ribosomes and mutant EF-G, will serve to further elucidate this mechanistic model.

It appears that the transient enhancements in the modification of bases A1408 and A1483, and of bases A1492 and A1493, that we observe can be directly or indirectly attributed to movement of domain IV of EF-G, using the posttranslocational position of EF-G as a model.¹ Since the movement of domain IV has been ascribed directly to the hydrolysis of GTP,⁴⁰ we propose that the alterations in chemical modification at these bases result directly from the movement of domain IV as GTP is hydrolyzed. Such movement apparently occurs as the two subunits are rotated with respect to one another.^{25,41,45} The transient enhancements observed at bases A1408 and A1483 and at bases A1492 and A1493 all peak out at ~30–45 ms. This is not evidence that GTP hydrolysis takes ~30–45 ms,^{36,55} but rather that the movement of domain IV of EF-G caused by GTP hydrolysis causes changes in that time period.

Specifically, we propose that movement of domain IV of EF-G mechanically stabilizes or pushes on base A1408 (through base G1494 of helix 44 and/or base A1913 of helix 69 of 23S rRNA–bridge B2a) and also on base A1493 (and possibly A1492), as GTP is hydrolyzed (see Fig. 6). We are not unaware that such movement of helix 44 in the decoding region is a direct result of the thermal oscillations occurring randomly in the ribosome between the classical and hybrid states.^{13,23} Our results suggest that such movement is stabilized in the rotated conformation by the binding of EF-G.

This results in a bending of helix 44 and a lateral movement of the proximal region of helix 44, estimated to be about 6–8 Å.^{41,45} The resultant bending and movement of the proximal region of helix 44 causes the hydrogen bond between N₁ of base A1483 and G1959 to be disrupted, as evidenced by its transient enhancement as GTP is added to 70S ribosomes with EF-G bound. During this, A1418 remains almost unchanged, showing only slight protection. Since there is no apparent direct or indirect interaction of either base A1418 or A1483 with EF-G, we concur that this site is an excellent candidate to be the hinge or pivot point as suggested by others.^{6,45} This pivoting could be either for the bending of helix 44 during the intersubunit rotation (ratcheting) or the swiveling process, with the A1418 platform being held in place by 23S rRNA from the 50S ribosomal subunit, while A1483 moves relative to this platform, or perhaps would also be involved in the swiveling of the head of the 30S ribosomal subunit.^{11,45}

Two bases that we studied in this region, A1413 and A1418, do not appear to be affected directly by domain IV movement. As noted previously (see Fig. 1), base A1413 appears to be directly in contact with base A909, which in turn is in contact with Lys17 of the N-terminal arm of protein S12. S12 is directly contacted by the globular portion of EF-G domain III, not domain IV. Thus, as EF-G is bound, A1413 is affected immediately, showing immediate protection, increasing somewhat when mRNA and tRNAs are present. There is no transient enhancement behavior here. This corroborates the observation of Valle *et al.*¹² that EF-G may act as a molecular wedge as it initially binds. A1418 and A1483 are distant from EF-G.

Thus, as EF-G is initially bound, induced-fit binding⁵⁶ or conformational selection²³ occurs, causing the A1413 protections, possibly due to the formation of hydrogen bonds between N₁ of A1413 and G1487. This is directly followed by movement at A1408, A1492, and A1493 induced by the structural change in domain IV of EF-G upon GTP hydrolysis, causing the proximal end of helix 44 to bend or to be stabilized in the rotated position. We postulate that the interaction of S12 with base A909 buttresses base A1413, providing a stable fulcrum for the bending of the proximal end of helix 44.

These results are depicted in the cartoon model (Fig. 6) in which the initial binding of EF-G causes some movement and/or stabilization of helix 44 as the globular portion of EF-G and the globular portion of S12 interact, immediately following which the hydrolysis of GTP causes domain IV of EF-G to move and interact with the proximal regions of helix 44 directly, or through bridge B2a with A1408, causing the ratcheting or twisting movement of helix 44. We propose that those bases (A1483, A1492, and A1493 and perhaps A1408) whose solvent environments are transiently enhanced are those directly or indirectly affected by the movement of domain IV of EF-G. We expect that additional bases in the proximal region of helix 44 and elsewhere are affected as well, but the DMS probing identifies only adenosines and the cytosines that have been modified.

Conclusions

This study represents a first effort to identify specific nucleotides that are involved in the translocational process and the approximate timing of their activity in ribosome functions.

The results show that this approach can identify individual nucleotides active in structural changes occurring in the ribosome and, in some cases, identify individual hydrogen bonds being made or broken in the process. Additional experiments using *T. thermophilus* ribosomes, mutant ribosomes, and antibiotics are underway. Many more time-dependent analyses of this process under carefully controlled conditions are needed to illuminate the molecular details of structural changes occurring in real time as translocation takes place, including ratcheting, the phases of intersubunit rotation, opening and closing, and head swiveling of the small subunit. Certainly, the ribosome is much more dynamic with exquisite timing and spatial resolution than we presently know. We have to concur completely with Roger Garrett who noted that the ribosome is “probably the most sophisticated machine ever made”.⁵⁷

Materials and Methods

Ribosome and 16S rRNA preparation

30S ribosomal subunits were prepared from *E. coli* MRE600 bacteria as previously described by Hennelly *et al.*³⁸ 70S ribosomes were activated by incubating at 42 °C for 20 min followed by 37 °C for 10 min. 16S RNA was extracted and purified exactly as described by Woolstenhulme and Hill.³⁹

Preparation of samples

70S ribosomes are activated by heating at 42 °C for 20 min, followed by 37 °C for 10 min in MB3 buffer (50 mM Hepes, 7 mM MgCl₂, 70 mM NH₄Cl, 6 mM β-mercaptoethanol, and 70 mM KCl). 70S –EF-G samples were prepared by adding apo-EF-G directly to 70S samples after 42 °C treatment and then incubating at 37 °C for 10 min. For 70S–mRNA–tRNAs, 1 μM mRNA was added directly to a 0.5-μM 70S sample after 42 °C activation and incubated for 15 min at 37 °C, following which 2 μM tRNA^{fMet} was added and incubated for 15 min at 37 °C, following which 2 μM Phe-tRNA was added and incubated with the mix at 37 °C for 15 min. EF-G-GTP was made by mixing 2 μM EF-G with 2 mM GTP at room temperature for 30 min. All experiments were run in MB3 buffer.

Time-dependent DMS chemical probing

For the first set of experiments, we placed 0.5 μM activated 70 S ribosomes in the first syringe of a BioLogic SFM-400 (BioLogic, USA) quench-flow apparatus. We placed 1 μM EF-G with 1 mM GTP in the second syringe and we mixed the two for predetermined aging times as designated in the tables. DMS [50% (v/v) DMS/ethanol] was added from the third syringe and followed by quench (75% β-mercaptoethanol, 25% propanol, 0.1 vol NH₄OAc, and 20 mM MgCl₂) after 5 ms, after which the sample was collected and analyzed by primer extension.⁴² These measurements were all made at 20 °C.

Equilibrium probing

Equilibrium probing of all of the above samples took place in the BioLogic quench-flow apparatus. Samples were brought to equilibrium on the bench for 30 min at 37 °C, following which the sample was inserted into syringe 1 of the BioLogic instrument and mixed briefly with buffer in syringe 2, following which DMS and quench were added, as with samples run in the time-dependent manner.

Analysis of probing experiments

After extracting samples with phenol 2×, phenol/chloroform 1×, and chloroform 3×, they were precipitated 2× in 5 M NH₄OAc and ethanol to remove excess GTP. Primer extension was used to visualize probed RNA samples and performed as previously described.⁵⁸

Analysis of these gels and quantification of band intensity were performed using a Fujifilm FLA 3000 phosphorimager (Fujifilm Corporation, Minato-ku, Tokyo, Japan) with ImageGauge Software. Normalization and quantification of the results were carried out as outlined previously.³⁷ Due to the imprecision of gel analysis, the relative intensities vary somewhat from gel to gel. We did average several values to obtain those reported. These values can also change depending on experimental conditions, which can make some difference in absolute values.

Structural analysis

Interactions were determined using Protein Data Bank (PDB) ID 2WRI¹ or as designated in the figure legends, in conjunction with PyMOL Software (DeLano Scientific). It must be remembered when comparing with crystal structures that most of these are from *T. thermophilus*, which varies from *E. coli* ribosome structures in some regions. Additionally, crystals are obtained under buffer conditions significantly different from those used in this study.

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Abbreviations used

rRNA	ribosomal RNA
DMS	dimethyl sulfate
PDB	Protein Data Bank
EF-G	elongation factor G

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Fig. 1. PyMOL (DeLano Scientific) representation of the proximal end of helix 44 of 16S rRNA with EF-G, protein S12, and helix 69 of 23S rRNA also present. (a) Portion of helix 44 showing bases A1492 and A1493 in conjunction with EF-G-domain IV and helix 69 of 23S rRNA. (b) Expanded view of A1408 in conjunction with helix 69. Base G1494 not shown (see the text). (c) Base A1413 in conjunction with base A909 and Lys21 of protein S12. (d) Expanded view of bases A1418 and A1483 in the posttranslocational position. These views are all taken from the posttranslocational position of the ribosome with EF-G-GDP present as reported by Gao *et al.* (PDB ID 2WRI).¹

Fig. 2.

Gel pattern of time-dependent studies showing modification of base A1483 when EF-GGTP and EF-G-GDP were added to the 70S ribosomes. Note that compared to the control 70S lanes, significant enhancement occurs as time increases and culminates in equilibrium values. Quantified values for this and other gels are shown in Table 1 and graphically in Figs. 3–5.

Fig. 3. Graph showing modification of bases noted when 70S ribosomes were mixed with EF-G-GTP for the delay times shown. The relative intensity is measured in Fuji units obtained from the ImageGauge software package of the Fujifilm phosphorimager. Error bars are shown as $\pm 10\%$ from the average values shown in Table 1 to allow for normalization errors. Aging times include delay time plus time of DMS mixing. Equilibrium times are shown as 10 s but were actually taken at 30 min.

Fig. 4. Graph showing the modification occurring in the first 45 ms after GTP was added to 70S–apo-EF-G complex. Error bars are shown as 10% of the values shown in Table 2. Equilibrium times are not shown on this graph but can be found in Table 2.

Fig. 5. Graph showing modification occurring when EF-G-GTP was added to a mix containing 70S ribosomes, mRNA, tRNA^{fMet}, and tRNA^{Phe}. Error bars are shown as 10% of the values shown in Table 1. Equilibrium times are shown as 10 s but were actually taken at 30 min.

Fig. 6. PyMOL (DeLano Scientific) cartoon representation of proposed biomechanical movement of the proximal end of helix 44 as EF-G is added. The basis for this structure is taken from the rotated state reported by Dunkle *et al.* (PDB ID 3R8N).⁴⁵ Arrows represent proposed movement due to the binding of EF-G or due to the movement of EF-G domain IV caused by GTP hydrolysis.

Table 1

Relative intensity values from primer extension gels for the six bases studied in the proximal region of helix 44 of 16S rRNA

Time (ms)	70S + EF-G with			70S, mRNA, tRNAs with			70S + EF-G with			70S, mRNA, tRNAs with		
	GTP	GDP	GDPNP	EF-G-GTP	EF-G-GDPNP	Time (ms)	GTP	GDP	GDPNP	EF-G-GTP	EF-G-GDPNP	Time (ms)
A1408						A1483						
0	100	100	100	100	100	0	100	100	100	100	100	
15	—	—	103	85	120	15	—	—	69	98	94	
30	56	106	94	89	118	30	170	168	78	110	95	
45	—	—	100	82	107	45	—	—	89	116	85	
90	55	103	—	65	—	90	207	197	—	122	—	
Equil.	44	91	106	67	76	Equil.	185	218	166*	116	139*	
A1413						A1492						
15	—	—	89	58	70	15	—	—	94	131	100	
30	63	105	86	58	90	30	50	94	99	121	113	
45	—	—	72	42	97	45	—	—	82	113	119	
90	65	113	—	40	—	90	49	90	—	131	—	
Equil.	55	126	95	47	97	Equil.	44	86	89	126	224*	
A1418						A1493						
15	—	—	84	86	82	15	—	—	93	104	113	
30	143	191	86	93	116	30	52	89	85	129	111	
45	—	—	94	79	130	45	—	—	—	110	96	
90	153	222	—	64	—	90	49	88	—	142	—	
Equil.	147	250	177*	81	104	Equil.	40	82	130*	150	216*	

In these experiments, EF-G-GTP, EF-G-GDP, and EF-G-GDPNP were added to 70S ribosomes and EF-G-GTP and EF-G-GDPNP were added to 70S ribosomes, mRNA, tRNA^{fMet}, and tRNA^{Phe} for the delay times shown. Samples were collected and the rRNA was extracted and run on sequencing gels after primer extension. Values given are average values for multiple gels on different runs.

Equilibrium (Equil.) values for EF-G-GDPNP marked with an asterisk (*) are anomalously high, as discussed in the text. Variations were within $\pm 10\%$.

Results are shown graphically in Figs. 3 and 5 for some of these experiments.

Table 2

70S–apo-EF-G with GTP added—70S–EF-G control

Time (ms)	A1408	A1413	A1418	A1483	A1492	A1493
0	100	100	100	100	100	100
15	122	82	81	120	187	169
20	112	84	80	129	191	179
30	108	85	93	151	206	230
45	84	83	84	114	139	103
Equil.	83	90	92	109	163	145

Relative intensity values from primer extension gels for the six bases studied in the proximal region of helix 44 of 16S rRNA. In this experiment, GTP was added to apo-EF-G bound to 70S ribosomes for the delay times shown. The design of this experiment was to identify the rapidity of modification changes occurring after GTP was added and hydrolyzed. 70S ribosomes with EF-G bound were used as control values. Note that three bases, A1483, A1492, and A1493, all show transient enhancement, peaking at about 30 ms. A1408 shows some immediate enhancement, followed by a reduction to equilibrium. Samples were collected and the rRNA was extracted and run on sequencing gels after primer extension. Values given are average values for multiple gels on different runs. Variations were within $\pm 10\%$. Results are shown graphically in Fig. 4.