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Influence of Guanine Nucleotides and Elongation Factors on Interaction of Release Factors with the Ribosome

(reticulocyte/fusidic acid/peptide chain termination)

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ABSTRACT **Release of formylmethionine from the reticulocyte ribosomal substrate, f[^{3}H]Met-tRNA·ribosome, is promoted by reticulocyte release factor (RF). The initial rate of this reaction is stimulated by GTP but inhibited by GDPCP. Formation of an RF·UA[^{3}H]A_2·ribosome complex is a measure of the binding of reticulocyte RF to the ribosome, and the recovery of this complex is increased by GDPCP and, to a lesser extent, GTP. These studies suggest that GTP is involved in the initial association of RF with the ribosome and that hydrolysis of the γ-phosphate of the guanine nucleotide is required at a subsequent rate-limiting step. The ribosomal-dependent fMet-tRNA hydrolysis and GTPase activities of reticulocyte RF are inhibited when elongation factor (EF)-2 is bound to the respective ribosomal substrate in the presence of fusidic acid and GDP. When EF-G is bound to the f[^{3}H]-Met-tRNA·AUG·ribosome substrate with fusidic acid and GDP, the fMet-tRNA hydrolysis activity of *Escherichia coli* RF-1 and RF-2 is also inhibited. The binding of reticulocyte RF and *E. coli* RF-1 or RF-2 to their respective ribosomes is prevented when fusidic acid·EF-2/EF-G·GDP·ribosome complexes are used.**

Peptide chain termination has been studied in mammalian extracts by slight modifications of the formylmethionine (fMet) release assay described for bacterial extracts (1, 2). Release of f[^{3}H]Met from reticulocyte f[^{3}H]Met-tRNA·ribosome substrates is directed by randomly ordered polynucleotides or by tetranucleotides of defined sequence containing the terminator codons (UAA, UAG, or UGA). This hydrolysis requires reticulocyte release factor, RF, and is stimulated by GTP. An RF fraction from rabbit reticulocytes was purified several hundred-fold and found to possess a ribosomal dependent GTPase that is stimulated by UAAA (3). Furthermore, this GTPase is stimulated by fusidic acid, a known antibiotic inhibitor of the ribosomal-dependent GTPase activities of prokaryotic and eukaryotic elongation factors (EF-G and EF-2) (3–6). Fusidic acid inhibits this intermediate step of protein biosynthesis indirectly by stabilizing the EF-G/EF-2·GDP·ribosome complexes (7–9). These stable complexes have been useful in studying the interaction of bacterial EF-G and aminoacyl-tRNA with the ribosome. Recent studies have shown that binding of EF-G to the bacterial ribosome prevents both nonenzymatic ribosomal binding of aminoacyl-tRNA and that catalyzed by EF-Tu (10–13); conversely, binding of aminoacyl-tRNA to the ribosome with or without EF-Tu prevents subsequent association of EF-G with these ribosomes (12, 14). Similarly, in mammalian extracts, EF-2 bound to the ribosome prevents the ribosomal binding of aminoacyl-tRNA catalyzed by EF-1 (15).

Since the ribosomal-dependent GTPase associated with reticulocyte RF is affected by fusidic acid, the ribosomal interactions of the release factors and elongation factors have been examined in this report. Formation of a reticulocyte RF·UA[^{3}H]A_2·ribosome complex has been used, in addition to reported assays for peptide chain termination (1, 2, 3, 18), to investigate the effects of guanine nucleotides and elongation factors on the interaction of RF with the ribosome.

Abbreviations: RF, release factor; EF, elongation factor.

MATERIALS AND METHODS

f[^{3}H]Met-tRNA was synthesized with L-[*methyl*-^{3}H]methionine (7.0 Ci/mmol, Amersham/Searle) and $tRNA_f^{Met}$ (*E. coli*) supplied by an interagency agreement through the National Institute of General Medical Sciences (NIGMS) (16). [γ-^{32}P]GTP (1.7 Ci/mmol) was obtained from Amersham/Searle, [^{3}H]GDP (9.5 Ci/mmol) and GDP from Schwarz/Mann, GTP from CalBiochem., GDPCP and poly(U,G,A) from Miles Laboratories, and fusidic acid from the Squibb Institute for Medical Research. Synthesis and purification of the oligonucleotides AUG, UGAA, UAGA, UAAA, UA[^{3}H]A_2, and UAA have been described (3, 17). Reticulocyte ribosomes were isolated, washed, and stored as described (1).

Conditions for RF-dependent f[^{3}H]Met-tRNA Hydrolysis on mammalian and bacterial f[^{3}H]Met-tRNA·ribosome/AUG·ribosome complexes have been described (2, 3).

Isolation of Reticulocyte RF·UA[^{3}H]A_2·Ribosome Complex. A typical reaction incubated for 20 min at 4° contained in 0.05 ml: 0.1 M ammonium acetate, 50 mM Tris-acetate (pH 7.4), 20 mM magnesium acetate, 1.8 A_{260} units of reticulocyte ribosomes, 15 μg of reticulocyte RF, 0.1 mM GDPCP, 20 pmol of UA[^{3}H]A_2 (6.0 Ci/mmol), and 20% (v/v) ethanol. The reaction mixture was brought to 0.5 ml with a dilution buffer containing 0.1 M ammonium acetate, 50 mM Tris-acetate (pH 7.4), 20 mM magnesium acetate, and 10 or 20% (v/v) ethanol. The complex was isolated on Millipore filters and washed with 25 ml of the dilution buffer. The RF·UA-[^{3}H]A_2·ribosome complex was quantitated by counting the radioactivity present on the dried filter in a POPOP–toluene scintillation fluid. Conditions for quantitating the binding of bacterial RFs to ribosomes was described (18).

Formation of Fusidic Acid·EF-G/EF-2·GDP·Ribosome Complexes. Reactions were incubated for 15 min at 4° and

contained typically in 0.05 ml: 10 mM Tris-chloride (pH 7.4), 10 mM magnesium chloride, 10 mM ammonium chloride, 1 μM GDP or 1 μM [^{3}H]GDP (9.5 Ci/mmol), 3 mM fusidic acid, either 1 A_{260} unit of reticulocyte ribosomes or 4 pmol of f[^{3}H]Met-tRNA·ribosome complex, and 23 μg of EF-2. Reactions studying bacterial reactions were identical except 2 pmol of f[^{3}H]Met-tRNA·AUG·ribosome complex or 1.9 A_{260} unit of *Escherichia coli* ribosomes and 38 μg of EF-G were used. The fusidic acid·EF-2/EF-G·GDP·ribosome complex was quantitated with [^{3}H]GDP on Millipore filters (9). Amounts of EF-2 and EF-G that gave maximum ribosomal binding of [^{3}H]GDP were used in all studies. These preformed ribosomal and f[^{3}H]Met-tRNA·ribosomal complexes were used with their homologous species of RF for studies of ribosomal binding, GTP hydrolysis, or fMet-tRNA hydrolysis.

Purification of RFs and EFs. The reticulocyte RF (Fraction V) used in these studies was purified as described (3), except phosphocellulose column chromatography was used after Sephadex G-200 column chromatography. This RF fraction obtained is free from EF-1 and EF-2. The *E. coli* release factors, RF-1 (fraction VI) and RF-2 (fraction VII), were purified as described (19). Isolation of reticulocyte EF-2 and bacterial EF-G were described (3, 20). Each is free from RF and EF-1/EF-Tu.

RESULTS

The activity of mammalian release factor can be determined in an assay of peptide chain termination with f[^{3}H]Met-tRNA·ribosome as substrate and randomly ordered RNA polymers or tetranucleotides of defined sequence as mRNA templates (3). The initial rate of f[^{3}H]Met release directed by oligonucleotides was increased by addition of GTP, inhibited by the analogue GDPCP (Fig. 1), and unaffected by GDP (not shown). With prolonged incubation (120 min, not shown) about 90% of the f[^{3}H]Met on the ribosomal substrate was released under the three conditions (Fig. 1). This stimulation of the initial rate of reaction by GTP was greatest (5-fold) when RF was present in small amounts (2 μg) and diminished as the concentration of RF was increased (Fig. 2). The rate of f[^{3}H]Met release in reactions containing GDPCP was inhibited at all concentrations of RF. It has been previously shown that GTP has no effect on either formation of f[^{3}H]-Met-tRNA·ribosome substrates under the conditions used (1) or its reactivity with puromycin. Since the reticulocyte RF fraction is devoid of EF-1 and EF-2, GTP apparently stimulates a rate-limiting step(s) of *in vitro* polypeptide chain termination.

TABLE 1. *Requirements for ribosomal UA[^{3}H]A$_2$ binding with reticulocyte RF*

Components	Δpmol of UA[^{3}H]A$_2$
Complete	2.06
−RF	0.07
−Ribosomes	0.02
−Ethanol	0.07
−GDPCP	0.40
−GDPCP, + GTP	0.85
−GDPCP, + GDP	0.43

Complete reactions contained components as described in *Methods*.

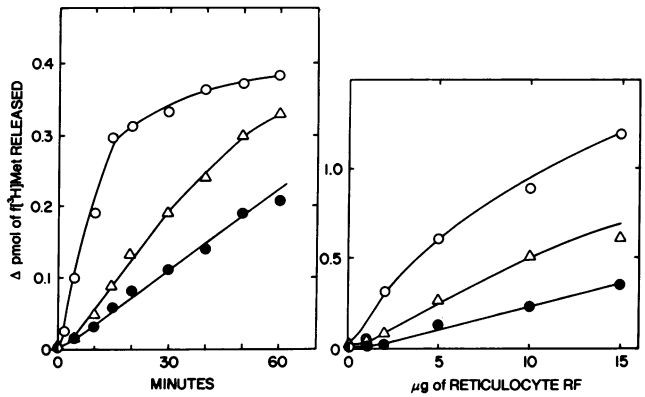

FIG. 1 (*left*). Effect of guanine nucleotides on time-dependent f[^{3}H]Met release. Reaction mixtures were incubated at 24° for the indicated time and contained in 0.05 ml: 0.75 pmol of f[^{3}H]-Met-tRNA·ribosome intermediate, 2.5 μg of reticulocyte RF, 0.1 A_{260} unit of UAAA, buffer components as described (3), and, as indicated, 0.1 mM GTP (○), 0.1 mM GDPCP (●), or no guanine nucleotide (△). The values obtained in the absence of UAAA (0.06–0.09 pmol) were subtracted.

FIG. 2 (*right*). Effect of guanine nucleotides on RF concentration-dependent f[^{3}H]Met release. Reaction mixtures were incubated at 24° for 15 min and contained in 0.05 ml: 2.0 pmol of f[^{3}H]Met-tRNA·ribosome intermediate, 0.1 A_{260} unit of UAAA, buffer components as described (3), 1–15 μg of reticulocyte RF and, as indicated, 0.1 mM GTP (○), 0.1 mM GDPCP (●), or no guanine nucleotide (△). The values obtained in the absence of UAAA (0.21–0.30 pmol) were subtracted.

Effects of GTP on partial reactions of polypeptide chain termination *in vitro* were investigated. Earlier studies indicated that bacterial RF-1 and RF-2 bind to ribosomes in response to trinucleotide codons and form stable RF·UA[^{3}H]A·ribosome complexes that can be quantitated by retention on Millipore filters (18). This method was modified for study of binding of reticulocyte RF to ribosomes. Since tetranucleotides, and not the trinucleotide codons, direct the release of f[^{3}H]Met from f[^{3}H]Met-tRNA·ribosome substrates, UA[^{3}H]A$_2$ rather than UA[^{3}H]A was used in all studies.

The requirements of ribosomes and RF for retention of UA[^{3}H]A$_2$ on Millipore filters (Table 1) indicated formation of an RF·UA[^{3}H]A$_2$·ribosome complex. This intermediate was stabilized by ethanol. The amount of complex isolated was markedly affected by the presence of guanine nucleotides. The recovery of RF·UA[^{3}H]A$_2$·ribosome was increased by GDPCP (5-fold) and GTP (2-fold), but not by GDP. While GTP and GDPCP both stimulate binding of RF to ribosomes, GTP hydrolysis is not essential since GDPCP is the more effective nucleotide. Thus, GDPCP is an inhibitor of the RF-dependent f[^{3}H]Met release but it stimulates recovery of RF·UA[^{3}H]A$_2$·ribosome complexes. GTP, however, stimulates the rate of f[^{3}H]Met release and recovery of the intermediate complex.

The codon recognition properties of reticulocyte RF were determined indirectly in Fig. 3 (*top*). In these studies the amount of RF present limited the quantity of RF·UA[^{3}H]A$_2$·ribosome formed. The addition of nonradioactive UAAA, UAGA, or UGAA competed with UA[^{3}H]A$_2$ for formation of RF·UA[^{3}H]A$_2$·ribosome complexes. A higher concentration of UAGA was required for complete competition (not shown)

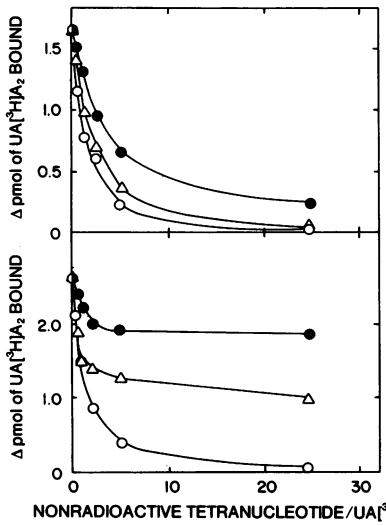

FIG. 3. Codon specificity of reticulocyte and *E. coli* RFs. (*Top*) Reactions contained 10.5 μg of reticulocyte RF, 2.7 A_{260} units of reticulocyte ribosomes, other components for formation of a reticulocyte RF·UA[³H]A_2·ribosome complex, and, as indicated, increasing amounts of UAAA (○), UGAA (△), and UAGA (●). (*Bottom*) Reactions contained 5.1 μg of *E. coli* RF-1, 5.9 μg of *E. coli* RF-2, 2.9 A_{260} units of *E. coli* ribosomes, other components for formation of bacterial RF·UA[³H]A_2·ribosome complex (18), and, as indicated, increasing amounts of UAAA (○), UGAA (△), and UAGA (●). Values obtained in the absence of RFs (0.17 pmol, reticulocyte study; 0.44 pmol, *E. coli* study) were subtracted in each case.

since it contained significant contamination of UAGp. These results obtained are consistent with recognition of all three terminator codons by reticulocyte RF. A similar study with *E. coli* ribosomes and a mixture of RF-1 and RF-2 is shown in Fig. 3 (*bottom*). UAGA competed with that part of the RF·UA[³H]A_2·ribosome complex due to RF-1, and UGAA that portion due to RF-2. The two bacterial RF species (RF-1, UAA, UAG; RF-2, UAA, UGA) are distinguished, therefore,

TABLE 2. *Requirements for EF-2 inhibition of reticulocyte RF release of formylmethionine*

Preincubation additions	Δpmol of f[³H]Met
None	0.57
GDP, EF-2, fusidic acid	0.27
EF-2	0.69
Fusidic acid	0.63
GDP	0.61
EF-2, fusidic acid	0.37
GDP, fusidic acid	0.64
GDP, EF-2	0.55

EF-2, GDP, and fusidic acid, as indicated, were first incubated with 4 pmol of f[³H]Met-tRNA·ribosome and other components as described in *Methods*. Release reaction mixtures were incubated for 15 min at 24° and contained in 0.05 ml: 8 μg of RF, 0.2 A_{260} unit of poly(U, G, A), 0.1 mM GTP, 0.02 ml of the preincubation reaction mixture, and buffer components as described (3). The value (0.49 pmol) obtained in the absence of UAAA was subtracted.

TABLE 3. *Requirements for EF-G inhibition of E. coli RF-1 release of formylmethionine*

Preincubation additions	Δpmol f[³H]Met	
	5 min	15 min
None	0.21	0.48
GDP, EF-G, fusidic acid	0.06	0.25
EF-G	0.23	0.46
Fusidic acid	0.20	0.50
GDP	0.22	0.51
EF-G, fusidic acid	0.24	0.44
GDP, fusidic acid	0.24	0.49
GDP, EF-G	0.22	0.50

EFG, GDP, and fusidic acid, as indicated, were incubated with 2 pmol of f[³H]Met-tRNA·AUG·ribosome and other components as described in *Methods*. Release reactions were incubated for either 5 or 15 min at 24° and contained in 0.05 ml: 6.5 μg of RF-1, 0.1 A_{260} unit of UAA, 0.02 ml of the preincubation reaction mixture, and buffer components as described (2). The values obtained in the absence of UAAA (0.12 and 0.15 pmol) were subtracted.

by this indirect approach. The indirect analysis of the codon recognition properties of reticulocyte RF is consistent with purification of RF from reticulocyte extracts (21). It appears that reticulocyte RF recognizes all three terminator codons and that binding of RF molecules to ribosomes can be measured by formation of an RF·UA[³H]A_2·ribosome complex.

The highly purified reticulocyte RF not only binds to ribosomes in response to GTP but, as previously reported, has a ribosomal-dependent GTPase activity that is stimulated by fusidic acid (3). Since EF-2 also possesses a ribosomal-dependent GTPase, which, in contrast, is inhibited by fusidic acid, we have examined the possible relationships between the interactions of EF-2 and RF with ribosomes. Fusidic acid stabilizes EF-G/EF-2·GDP·ribosome complexes (7–9). In the following studies, peptide chain termination *in vitro* has been examined with ribosomal substrates containing stabilized EF-G or EF-2.

The time-dependent release of f[³H]Met from the f[³H]Met-tRNA·ribosome substrate previously incubated with or without EF-2, GDP, and fusidic acid is given in Fig. 4. The rate of release of f[³H]Met from the substrate previously incubated with EF-2, GDP, and fusidic acid was 85% inhibited at 5 min. The f[³H]Met release observed with ribosomes complexed with EF-2 does not necessarily indicate that RF can function on such substrates. The GDP in the fusidic acid·EF-2·[³H]GDP·ribosome complex exchanges slowly under these assay conditions. The rate of release from inhibition might be influenced by an active competition between RF and EF-2 for a ribosomal site. The requirements for EF-2 inhibition of RF-dependent fMet-tRNA hydrolysis are shown in Table 2. The 15-min time of incubation used in this study does not represent the optimal inhibition of release (Fig. 4). Single additions of EF-2, fusidic acid, or GDP, or combined additions of GDP and fusidic acid, or GDP and EF-2 did not inhibit f[³H]Met release. Inhibition of RF-mediated fMet-tRNA hydrolysis occurred, however, when EF-2 was prebound as fusidic acid·EF-2·GDP ribosomal complex. Although GTP and GDPCP will also form those complexes, we have used GDP in these studies since it has no effect on peptide chain termination. Thus the partial inhibition of

TABLE 4. *Requirements for EF-2 inhibition of reticulocyte RF-dependent GTP hydrolysis*

Preincubation additions	Δpmol of [γ-^{32}P]GTP hydrolyzed +RF	−RF
GDP, fusidic acid, ribosomes	93.9	3.0
EF-2, GDP, fusidic acid, ribosomes	26.9	22.1
EF-2, fusidic acid, ribosomes	33.8	—
EF-2, GDP, ribosomes	64.5	36.0
EF-2, ribosomes	63.2	—
Fusidic acid, ribosomes	93.9	—
GDP, ribosomes	61.9	—
Ribosomes	61.3	3.0
None	(4.7)	(1.7)

EF-2, GDP, and fusidic acid, as indicated, were first incubated with 1 A_{260} unit of ribosomes and other components as described in *Methods*. Reaction mixtures were incubated for 10 min at 24° and contained in 0.05 ml: 8 μM [γ-^{32}P]GTP, and, as indicated, 6 μg of reticulocyte RF, 0.02 ml of preincubation reaction mixture, and buffer components as described (3). The values for the [γ-^{32}P]GTP hydrolysis with and without RF in the absence of ribosomes (*in parentheses*) were subtracted as appropriate.

TABLE 5. *Requirements for EF-2/EF-G inhibition of the binding of reticulocyte RF/E. coli RF-2 to ribosmes*

Preincubation addition	Δpmol of UA[^{3}H]A_2 Reticulocyte	*E. coli*
None	0.65	0.86
GDP, EF-2/EF-G, fusidic acid	0.07	0.16
GDP, fusidic acid	0.57	0.76
GDP, EF-2/EF-G	0.22	0.71
EF-2/EF-G, fusidic acid	0.22	0.85
EF-2/EF-G	0.26	0.91
Fusidic acid	0.62	0.86
GDP	0.63	0.82

EF-2/EF-G, GDP, and fusidic acid, as indicated, were first incubated with 2.5 A_{260} units of reticulocyte ribosomes or 1.9 A_{260} units of *E. coli* ribosomes and other components as described in *Methods*. Reaction mixtures were incubated 20 min at 4° and contained in 0.05 ml: 15 μg of reticulocyte RF or 40 μg of *E. coli* RF-2, 0.015 ml of the respective preincubation reaction mixture, and other components for formation of reticulocyte RF·UA-[^{3}H]A_2·ribosome complex as described in *Methods* or the corresponding *E. coli* complex (18). The values obtained in the absence of reticulocyte RF (0.25 pmol) and *E. coli* RF-2 (0.44 pmol) were subtracted.

f[^{3}H]Met release observed with EF-2 and fusidic acid in the absence of added GDP probably reflects a fusidic acid·EF-2·GDP·ribosome complex, formed after addition of GTP for assay of the release activity of RF.

As discussed above, fusidic acid stablizes EF-G binding to *E. coli* ribosomes as a fusidic acid·EF-G·GDP·ribosome complex. The interaction between release factors and elongation factors on ribosomes from prokaryotic and eukaryotic cells can be compared. As shown in Table 3, formation of a stable complex of fusidic acid·EF-G·GDP on the f[^{3}H]Met-tRNA·AUG·ribosome substrate significantly inhibited f[^{3}H]-Met release by RF-1. The inhibition was greatest at early time intervals, as in the reticulocyte studies. Essentially identical results were obtained when RF-2 was used (data not shown). Studies in both prokaryotic and eukaryotic cells indicate that binding of elongation factors to the ribosome inhibits the release activity of RF.

Hydrolysis of the γ-phosphate of GTP has been examined in reactions containing both EF-2 and RF, since both exhibit ribosomal-dependent GTPase activities that are inversely affected by fusidic acid. As shown in Table 4, the ribosomal-dependent GTPase activity of RF was *stimulated* by fusidic acid by 50% while the EF-2 GTPase activity was *inhibited* about 40% under identical conditions. In reactions containing both RF and EF-2, but not fusidic acid, the amount of [γ-^{32}P]-GTP hydrolyzed was 64 pmol rather than the 98 pmol expected if the two activities were additive. In reactions containing fusidic acid, GDP, EF-2, and RF, the amount of GTP hydrolyzed (26.9 pmol) was 20% of the expected sum of the RF and EF-2 activities (130.0 pmol). Since GTP is a component of the GTPase assay, significant inhibition was also observed with the same components if GDP were omitted. These studies suggest, therefore, that EF-2 in a stable association with ribosomes inhibits the RF ribosomal-dependent GTPase activity. Furthermore, the lack of additive GTPase activities of the protein factors in the absence of fusidic acid suggests that a common rate-limiting step is involved in both activities or that both RF-2 and EF-2 cannot function simultaneously.

As shown in the preceding studies, formation of fusidic acid·EF-2·GDP·ribosomal subtrate complexes inhibits both the RF-dependent f[^{3}H]Met release and GTPase activities on reticulocyte ribosomes. Furthermore, the equivalent *E. coli* ribosomal complexes formed with EF-G inhibit RF-1- and RF-2-dependent f[^{3}H]Met release. Each of these reactions require interaction of RF with the ribosome, and inhibition of this interaction would result in loss of RF functions. Binding of bacterial and mammalian RF to their respective ribosomes, both with or without fusidic acid·EF-2/EF-G·GDP, was directly examined. The interaction of bacterial and

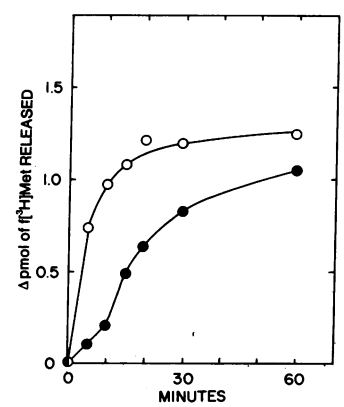

FIG. 4. EF-2 inhibition of RF release of f[^{3}H]Met. Release reaction mixtures were incubated at 24° for the indicated time and contained in 0.05 ml: 8 μg of RF, 0.1 A_{260} units of UAAA, 0.1 mM GTP, buffer components as described (3), and either 2.0 pmol of f[^{3}H]Met-tRNA·ribosome intermediate (○) or 2.0 pmol of f[^{3}H]Met-tRNA·ribosome intermediate, previously incubated with EF-2, GDP, fusidic acid, and buffer components (●). The value obtained in the absence of UAAA (0.65 pmol) was subtracted.

reticulocyte RF with ribosomes was determined by formation of RF·UA[^{3}H]A_2·ribosome complexes.

Binding of reticulocyte RF was significantly inhibited when preformed fusidic acid·EF-2·GDP·ribosome complexes were used (Table 5). Since GDPCP is added to reactions for maximum recovery of the RF·UA[^{3}H]A_2·ribosome complex, the partial inhibition observed in all other reactions containing EF-2 could be a result of stable EF-2·GDPCP·ribosome complexes formed during binding of RF. GDPCP forms stable ribosomal complexes with EF-2 (22). Preformation of the fusidic acid·EF-2·GDP·ribosome complex resulted in greater inhibition of RF binding (90%) than that given by the analogous GDPCP complexes (65%). Similar studies with *E. coli* ribosomes and the *E. coli* release factor, RF-2, are also shown in Table 5. Formation of a fusidic acid·EF-G·GDP·ribosome complex inhibited binding of RF-2 to *E. coli* ribosomes. These studies were repeated with *E. coli* RF-1 (not shown), and the interaction of RF-1 with the fusidic acid·EF-G·GDP·ribosome complex was also inhibited. In both prokaryotic and eukaryotic cells, therefore, binding of release factors to ribosomes is prevented if elongation factors are associated with the ribosome.

DISCUSSION

Stimulation of the initial rate of RF-dependent fMet-tRNA hydrolysis on reticulocyte ribosomal substrates by GTP, greatest at low concentrations of RF, and inhibition of this reaction by GDPCP suggest that an intermediate event of peptide chain termination *in vitro* related to GTP hydrolysis is rate limiting. Since release of f[^{3}H]Met is a measurement of all intermediate events of peptide chain termination *in vitro*, the guanine nucleotide effects on partial events of this process were examined. We found that guanine nucleotides affect the interaction of RF with the ribosome. The recovery of reticulocyte RF·UA[^{3}H]A_2·ribosome complexes was increased 2-fold with GTP, 5-fold with GDPCP, and was unaffected by GDP. These studies suggest that interaction of RF with the ribosome requires GTP, while hydrolysis of the γ-phosphate of the guanine nucleotide is required for the maximum rate of f[^{3}H]Met release. This hypothesis is analogous to other intermediate events of protein biosynthesis. Initiation factor-dependent fMet-tRNA binding to ribosomes (23) and EF-Tu- or EF-1-dependent binding of aminoacyl-tRNA to ribosomes (24, 25) is stimulated both by GTP and GDPCP, but only GTP promotes the participation of these molecules in peptide chain initiation and elongation. Furthermore, binding of EF-G or EF-2 to ribosomes is promoted by GTP or GDPCP, but complexes formed with GDPCP are not translocated (22, 26).

An earlier report discussed guanine nucleotide effects on polypeptide chain termination *in vitro* in *E. coli* extracts (27). An *E. coli* factor, RF-3, stimulates the rate of f[^{3}H]Met release with RF-1 and RF-2 by increasing their binding to ribosomes. Furthermore, RF·UA[^{3}H]A_2·ribosome complexes formed with RF-3 dissociate rapidly with addition of GDP, GTP, or GDPCP. Thus, RF-3 can promote RF-1 or RF-2 binding to the ribosome and, through its interaction with the above guanine nucleotides, destabilize this association. GTP stimulation of *E. coli* RF-1 or RF-2 binding to ribosomes in reactions containing RF-3 has not been demonstrated. Conversely, an RF-3-like activity has not been separated from reticulocyte extracts. The differences in the characteristics of RF binding to ribosomes *in vitro* in *E. coli* and reticulocyte extracts is currently under investigation. The following observations are taken from studies with reticulocyte and *E. coli* extracts: (*i*) Reticulocyte RF binding to ribosomes is stimulated by GTP but does not require its hydrolysis; (*ii*) a ribosomal-dependent GTPase activity is associated with reticulocyte RF; (*iii*) the effect of GTP hydrolysis is greatest on *in vitro* chain termination when RF is limiting; (*iv*) *E. coli* RF-1 or RF-2·UA[^{3}H]A_2·ribosome complexes formed with RF-3 are destabilized by GDP. Collectively these observations suggest that peptide chain termination *in vitro* involves interaction of RF with the ribosome requiring GTP, followed by hydrolysis of the GTP on the ribosome, and, finally, destabilization of RF as a result of the generation of GDP thereby allowing the RF to recycle. This model appears to accommodate the available data from studies of both cellular extracts. Models with similar features have been proposed for the bacterial initiation factor IF-2 (28, 29) and elongation factors EF-Tu (30–32) and EF-G (5).

Binding of reticulocyte RF and *E. coli* RF-1 and RF-2 to their ribosomes can be inhibited by formation of ribosomal complexes saturated with the elongation factors, EF-2 and EF-G, respectively. Exclusion of reticulocyte RF from the ribosome by the elongation factors inhibited the hydrolysis of GTP and fMet-tRNA. Other studies have shown that stable EF-G binding to the *E. coli* ribosome can exclude the ribosomal binding of aminoacyl-tRNA with and without EF-Tu (10–13). Conversely, ribosomes containing aminoacyl-tRNA, bound enzymically with EF-Tu or nonenzymically, will not associate with EF-G (14). Similar studies with mammalian extracts have indicated that EF-2 bound to ribosomes with GDPCP inhibits the EF-1-dependent binding of aminoacyl-tRNA to these ribosomes (15). Collectively the studies indicate that EF-G and EF-2 can exclude the release factors (RF-1, RF-2 in *E. coli* and RF in mammalian cells) and aminoacyl-tRNA from the ribosome. It has been assumed that since aminoacyl-tRNA binding to ribosomes is prevented by EF-G, EF-Tu is also excluded (11, 12). Furthermore, the involvement of the factors in the intermediate steps of protein biosynthesis may require not only their binding but also displacement from the ribosome. In *E. coli*, either RF-1 or RF-2 can bind to the ribosome and participate in chain termination, while studies reported here indicate that only a single reticulocyte RF recognition molecule is involved in ribosomal binding.

While a simple overlapping or identical site model may explain why two factors cannot be accommodated simultaneously on a single ribosome, several other possibilities exist. An alternative model, for example, could involve a single ribosomal event common to all three partial reactions of protein biosynthesis involving elongation and termination factors. This, in fact, would accommodate the need for a recycling mechanism for the interaction of the soluble factors (EF-1, EF-2, RF—reticulocyte; EF-Tu, EF-G, RF-1, RF-2—*E. coli*) with the ribosome. One enzyme function common to all three types of factors in reticulocyte extracts is their ribosomal-dependent GTPase activity. Further study is required for these partial reactions in *E. coli* extracts. Since several methods have been used successfully to study the ribosomal GTPase reactions in *E. coli* extracts (33–35), several approaches are available to resolve this question.

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