Binding of Met-tRNA<sub>f</sub> and GTP to Homogeneous Initiation Factor MP

(Received for publication, March 26, 1975)

BRIAN SAFER, SHERRILL L. ADAMS, W. FRENCH ANDERSON, AND WILLIAM C. MERRICK

From the Molecular Hematology Branch, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland 20014

Homogeneous initiation factor MP forms a stable complex with Met-tRNA<sub>f</sub> which binds to nitrocellulose filters in the absence of ribosomal subunits. Complex formation is rapid at 0°C and the rate of reaction is stimulated 20-fold by GTP when freshly prepared initiation factor MP is used. Under optimal assay conditions, a 1:1:1 stoichiometry for initiation factor MP, GTP, and Met-tRNA<sub>f</sub> is indicated, based on a molecular weight for initiation factor MP of 180,000. Kinetic analysis of ternary complex formation suggests an ordered reaction sequence with binding of GTP followed by binding of Met-tRNA<sub>f</sub>. However, binding of GTP appears to produce an unstable state which leads to rapid inactivation of initiation factor MP in the absence of Met-tRNA<sub>f</sub>. Formation of a stable binary complex of initiation factor MP and Met-tRNA<sub>f</sub> occurs in the absence of GTP. The binary complex cannot subsequently bind GTP. While storage of initiation factor MP at 0°C for several weeks has no effect on the rate or extent of Met-tRNA<sub>f</sub> binding in the presence of GTP, the rate of binary complex formation is increased 10-fold. The binary and ternary complexes appear to bind to 40 S ribosomal subunits with equal efficiency.

Several laboratories have partially purified two distinct eucaryotic protein factors from 0.5 M KCl ribosomal extracts which appear to be required for initiation complex formation (1-12). IF-M1,<sup>1</sup> recently purified to homogeneity (2), can bind initiator Met-tRNA<sub>f</sub>, to the 40 S ribosomal subunit in the presence of template (AUG or mRNA) and IF-M2B (13). In contrast, a second factor which we call IF-MP forms a stable ternary complex with Met-tRNA<sub>f</sub> and GTP. This ternary complex appears to bind to 40 S ribosomal subunits in the absence of messenger RNA or to nitrocellulose filters in the absence of ribosomal subunits (9-18). Partially purified fractions containing this factor have also been shown: (a) to reverse inhibition of globin mRNA translation produced by hemin deficiency (19) and double-stranded RNA (19, 20); (b) to bind to synthetic poly(A) and natural mRNA (18); and (c) to be required for formation of the 80 S initiation complex (9-12, 17, 21-23). In this report, optimal conditions required for kinetic studies of Met-tRNA<sub>f</sub> binding by homogeneous IF-MP have been determined (see accompanying paper (24) for purification and characterization of IF-MP). These studies show that the ternary complex, composed of equimolar IF-MP·GTP·Met-tRNA<sub>f</sub>, based on a molecular weight of 180,000 for IF-MP, is formed by an ordered sequential mechanism. In the absence of GTP, however, IF-MP can form a stable binary complex with Met-tRNA<sub>f</sub>, which binds to 40 S ribosomal subunits with equal efficiency as the ternary complex.

EXPERIMENTAL PROCEDURES

Materials—GTP, dithiothreitol, aurintricarboxylic acid, and N-ethylmaleimide were obtained from Calbiochem. Ribonuclease-free sucrose, [3H]methionine, [14C]methionine, and [3H]phenylalanine were from Schwarz/Mann. [35S]Methionine, [32P]GTP, [32P]GTP, and [8-3H]GTP were obtained from New England Nuclear. Unfractionated rabbit liver tRNA was purchased from General Biochemicals and rat liver tRNA<sub>30</sub> (37% pure) was obtained from Biogenics Research Corp. All other materials used were of reagent grade quality.

Preparation of IF-MP—Homogeneous IF-MP was prepared from the 0.5 M KCl extract of reticulocyte ribosomes as described in the preceding paper (24). This preparation of IF-MP contains no detectable methionyl-tRNA synthetase activity (data not shown). The experimental data reported in this paper were all obtained with unfrozen IF-MP within 7 days of preparation except when specifically stated otherwise. Although total (GTP-dependent and GTP-independent) Met-tRNA binding is stable for 1 week when stored at 0°C in Buffer A (20 mM Tris, pH 7.5/100 mM KCl/1 mM dithiothreitol/0.1 mM EDTA/10% glycerol), the rate of GTP-independent Met-tRNA binding is increased by prolonged storage under these conditions or at the vapor temperature of liquid N<sub>2</sub> (see "Results").

Preparation of Met-tRNA<sub>f</sub>—Rabbit liver tRNA<sub>30</sub> (13% pure), prepared by RPC-V chromatography of unfractionated tRNA (25), and rat liver tRNA<sub>30</sub> (37% pure) were acylated with [35S]methionine (12,000 to 14,000 cpm/pmol) or [14C]methionine (90 to 460 cpm/pmol) as previously described (26). Identical results were obtained with Met-tRNA<sub>f</sub> preparations acylated with [14C]- or [35S]methionine. The trichloroacetic acid precipitability of [14C]- or [35S]methionine in these preparations was 80 to 90%. Acylation of Met-tRNA<sub>f</sub> was 50 to 80% of that predicted. Met-tRNA<sub>f</sub> preparations were lyophilized as small aliquots and stored in liquid nitrogen until needed.

<sup>1</sup>The abbreviations used are: IF, initiation factor; GMP-P(CH<sub>2</sub>)<sub>2</sub>P, 8-y-methylene-guanosine triphosphate; binary complex, a complex of IF-MP and Met-tRNA<sub>f</sub>; ternary complex, a complex of IF-MP·GTP·Met-tRNA<sub>f</sub>; IF-MP*, an altered state of IF-MP which does not bind GTP.
Preparation of Unfractionated Reticulocyte Aminoacyl-tRNA—Unfractionated reticulocyte tRNA was acetylated with 16 ^{14}C-amino-acids and four ^{3}H-amino-acids using crude rabbit reticulocyte aminoacyl-tRNA synthetase prepared as described previously (26). The 6 ml of reaction mixture contained: 100 mM Tris-HCl, pH 7.5; 20 mM KCl; 5 mM MgCl₂; 1 mM dithiothreitol; 2 mM ATP; 20 PM concentration of each amino acid; 60 Aₘₚ units of unfractionated tRNA; and 1 mg of reticulocyte aminoacyl-tRNA synthetase. Following a 20-min incubation at 37°C, a 1/6 volume of 20% potassium acetate, pH 5.0, was added and the reaction was rapidly chilled to 0°C. An equal volume of liquid phenol was added and the solution was mixed vigorously for 5 min. After separation of phases by centrifugation, the aqueous phase was applied to a Sephadex G-25 column equilibrated with 0.1 M potassium cacodylate, pH 5.0. Fractions containing aminoacyl-tRNA were pooled, concentrated by lyophilization, and stored in liquid nitrogen until needed.

Preparation of Other Aminoacyl-tRNA—[^14]C-Phe-tRNA (800 cpm/pmol) was prepared from unfractionated rabbit liver or Escherichia coli tRNA which had been aminoacylated by an homologous enzyme as described previously (2, 26).[^3]H[Met-tRNAₘₚ (1,250 cpm/pmol) was prepared in the same manner as[^3]HMet-tRNAₘₚ using a crude reticulocyte synthetase preparation.

Binding of Binary and Ternary Complexes to 40 S Subunits—The preparation of 40 S ribosomal subunits from reticulocyte ribosomes washed with 0.5 M KCl was performed as previously published (13). The 40 S subunits were free of 60 S subunits as determined by lack of polyphenylalanine synthesis without addition of 60 S subunits to the assay system (2), and by the presence of only 18 S rRNA in 2% sodium dodecyl sulfate gels (57). Formation and binding of binary and ternary complexes of IF-MP to 40 S ribosomal subunits were performed as stated in the legend to Fig. 8. IF-MP-40 S complexes formed in this manner were stabilized by formaldehyde fixation according to the procedure of Clemens et al. (10).

Met-tRNA, Binding Assay—Met-tRNAₘₚ binding by IF-MP was conducted in 50 ml of reaction mixture containing: 20 mM Tris-HCl, pH 7.5; 50 mM KCl; and 0.6 mM dithiothreitol. When present, GTP was 10 PM. Since GTP-dependent Met-tRNAₘₚ binding activity is rapidly inactivated by preincubation of IF-MP with GTP, and IF-MP can form stable binary complexes with Met-tRNAₘₚ in the absence of GTP (see "Results"), binding reactions were initiated by the addition of protein to the assay buffer containing these ligands. Reaction mixtures were blended on a Vortex mixer before and after addition of IF-MP and incubated for the indicated times at 0°C. Complex formation was terminated by dilution with 2 ml of wash buffer of the following composition: 20 mM Tris-HCl, pH 7.5; 50 mM KCl; 10 mM MgCl₂; and 1 mM L-methionine. Immediately following dilution, the solution was passed through a nitrocellulose filter (Millipore, type HA). The filters were washed with three additional 2-ml aliquots of wash buffer and then dissolved, and bound radioactivity was determined in 10 ml of scintillation fluid (Instabray, Yorktown Research). Counting efficiency of ^{3}H and ^{14}C was 80%, of ^{3}H was 100%, and of ^{3}H was 20% using a Packard Tri-Carb liquid scintillation spectrometer.

RESULTS

Characterization of Met-tRNA Binding Assay IF MP forms a stable ternary complex with initiator Met-tRNAₘₚ and GTP; this complex binds to nitrocellulose filters (9-18). Conditions required for maximal formation and binding of IF-MP-GTP-Met-tRNAₘₚ were determined. The requirement for potassium is optimal over a broad concentration range, 40 to 80 mM (data not shown). Precipitation of IF-MP occurred below 20 mM KCl. The pH optimum is 7.15 (data not shown). However, since other assay systems developed for the analysis of protein synthesis initiation (28) show pH optima at 7.5, a pH of 7.5 has been used in this study even though binding efficiency is reduced by approximately 15 to 20%. The concentration of GTP required for maximal extent of binding decreases with increasing IF-MP concentration (data not shown).

Since substrate inhibition is not observed, a GTP concentration which saturates at all input levels of IF-MP, 10 PM, is used in all binding assays. Exogenous Mg^{2+} is not required and has little effect on ternary complex formation from 0 to 10 mM Mg^{2+} (data not shown). In contrast, inclusion of Mg^{2+} in buffer used to wash the nitrocellulose filters after binding of the ternary (or binary) complexes appears to reduce nonspecific binding observed with nonhomogeneous preparations of IF-MP. With IF-MP partially purified through phosphocellulose chromatography (13), both GTP-dependent and GTP-independent Met-tRNAₘₚ binding are reduced an equal amount; as a result, dependency of the binding assay on GTP appears to be increased 4-fold (Fig. 1). Although it is important to distinguish between ternary complex formation and subsequent retention on nitrocellulose filters, with homogeneous IF-MP neither process is affected by Mg^{2+} concentrations as high as 10 mM.

Under these assay conditions the extent of GTP dependent Met-tRNAₘₚ binding increases linearly as a function of IF-MP over the range 0.1 to 1 µg of IF-MP/50 µl of binding assay (Fig. 2). Similar results are obtained at 5 mM Mg^{2+} in the presence of IF-MP.
or absence of salt-washed ribosomes or ribosomal subunits (data not shown).

Time Course of Met-tRNA<sub>i</sub> Binding—The time course of Met-tRNA<sub>i</sub> binding by homogeneous IF-MP is presented in Fig. 3A. At 0°, GTP-dependent binding is essentially complete by 1 min. In the absence of GTP, a much slower rate of Met-tRNA<sub>i</sub> binding is obtained. Although linear for the first 5 min of incubation, the extent of the GTP-independent Met-tRNA<sub>i</sub> binding is approximately 50 to 60% of the GTP-dependent Met-tRNA<sub>i</sub> binding. Both the ternary IF-MP-GTP-Met-tRNA<sub>i</sub> and the binary IF-MP-Met-tRNA<sub>i</sub> complexes are stable for up to 2 hours at 0° under these assay conditions and no deacylation is detected (data not shown). To measure initial rates for kinetic studies, GTP-dependent Met-tRNA<sub>i</sub> binding is determined at 15-s incubation at 0°, while GTP-independent binding is monitored at 5 min. The maximal extents of GTP-dependent and GTP-independent Met-tRNA<sub>i</sub> binding are obtained at 0 and 15 min, respectively. While the extent of Met-tRNA<sub>i</sub> binding at 30 or 37° is similar to that obtained at 0°, initial rates of complex formation were too rapid for accurate determination by the nitrocellulose filtration technique used in this study (data not shown).

Titrations of IF-MP Binding with Met-tRNA<sub>i</sub>—Titration of the extent of IF-MP binding with Met-tRNA<sub>i</sub> in the presence or absence of GTP (Fig. 3B) shows that saturation is achieved at 30 pmol/50 μl reaction (600 nm). Storage of IF-MP at 0° for 2 weeks or freezing has no effect on the extent (or rate) of Met-tRNA<sub>i</sub> binding in the presence of GTP; however, both the rate and extent of GTP-independent binding are doubled. As the result, GTP dependence of Met-tRNA<sub>i</sub> binding decreases. From data presented in Fig. 3A, GTP dependence is also greatly affected by the length of incubation. In contrast to a 4-fold dependency obtained at 4 min, a 20-fold dependency of the initial rates of Met-tRNA<sub>i</sub> binding is obtained at 15 s. The ability of the other common ribo or deoxyribonucleotide triphosphates to substitute for GTP has been studied. Preliminary results indicate that dGTP is as effective as GTP while the six other nucleotide triphosphates were less than 5% as active in stimulating complex formation (at concentrations of up to 20 μM).

Specificity of Met-tRNA<sub>i</sub> Binding by IF-MP—IF-MP binds the initiator Met-tRNA<sub>i</sub> to extent 3 times more efficiently than the isoaccepting species Met-tRNA<sub>S</sub> which is required for internal translation (Table I, 1 and 2). However, unlike IF-M1 which binds both Met-tRNA<sub>i</sub> and Phe-tRNA<sub>i</sub> (2), IF-MP does not recognize either rabbit liver or Escherichia coli Phe-tRNA<sub>i</sub> (Table I, 3 and 4). In addition IF-MP does not appear to recognize other aminoacyl-tRNA species (Table I, 5 and 6). In the presence of limiting unfractionated aminoacyl-tRNA (8 pmol), IF-MP binds only a level of aminoacyl-tRNA equivalent to the level of Met-tRNA<sub>i</sub> present in the mixture (0.2 pmol). In the presence of saturating levels of aminoacyl-tRNA (130 pmol), IF-MP binds a level of [<sup>14</sup>C]aminoacyl-tRNA equivalent to that obtained with purified Met-tRNA<sub>i</sub>. Also, the inclusion of unfractionated and uncharged reticulocyte tRNA did not alter the rate or extent of Met-tRNA<sub>i</sub> binding (data not shown), indicating that tRNA<sub>i</sub> species (as well as other uncharged species) do not compete with Met-tRNA<sub>i</sub> for binding to IF-MP.

Kinetic Parameters of Met-tRNA<sub>i</sub> Binding by IF-MP—Initial velocity data of GTP-dependent Met-tRNA<sub>i</sub> binding by IF-MP were analyzed by double reciprocal plots (Fig. 4, A and B). Initial rates were measured by the binding of Met-tRNA<sub>i</sub> to IF-MP. With the 37% pure preparation of rat liver Met-tRNA<sub>i</sub>, an apparent K<sub>m</sub> of 0.2 μM and V<sub>max</sub> of 15 pmol/μg/min were obtained for Met-tRNA<sub>i</sub>, while corresponding values for GTP were 0.5 and 15, respectively. Less purified preparations of Met-tRNA<sub>i</sub> (rabbit liver; 13%) depress both the rate and extent of IF-MP binding.

**Table I** Specificity of aminoacyl-tRNA binding by IF-MP

| Aminoacyl-tRNA species | -GTP | +GTP | -GTP | +GTP |
|-----------------------|------|------|------|------|
|                       | pmol bound/μg IF-MP | pmol bound/μg IF-MP | pmol bound/μg IF-MP | pmol bound/μg IF-MP |
| Met-tRNA<sub>i</sub> | 1.8 | 6.0 | 1.6 | 2.5 |
| Met-tRNA<sub>S</sub> | 0.6 | 4.1 | 0.3 | 0.8 |
| Phe-tRNA | 0.0 | 0.0 | 0.0 | 0.0 |
| Phe-tRNA (Escherichia coli) | 0.0 | 0.0 | 0.0 | 0.0 |
| Unfractionated aminoacyl-tRNA (8 pmol) | 0.1 | 0.1 | 0.2 | 0.2 |
| Unfractionated aminoacyl-tRNA (130 pmol) | 0.5 | 2.9 | 2.9 | 2.9 |
extent of ternary complex formation. This inhibition is uncompetitive with respect to Met-tRNA\(_A\) (Fig. 4A) and noncompetitive with respect to GTP (Fig. 4B). While the agent responsible has not been identified, this pattern of inhibition suggests that formation of the ternary complex proceeds by an ordered mechanism (29, 31), with compulsory binding of GTP preceding binding of Met-tRNA\(_A\). It is possible that the differences in the Met-tRNA\(_A\)s may reside in the source of the tRNA either as species specific (rat versus rabbit) or commercial supplier.

Stoichiometry of IF-MP-GTP-Met-tRNA\(_A\) Complex—To examine the stoichiometry of GTP and Met-tRNA\(_A\) in the ternary complex, a series of double label binding studies were performed using \([{^8}_{\text{H}}\text{GTP}}\), \([\alpha-^{32}\text{P}]\text{GTP}}\), or \([\gamma-^{32}\text{P}]\text{GTP}}\) and \([^{14}\text{C}]\text{Met-tRNA}}\). The level of both GTP and Met-tRNA\(_A\) binding appears to increase proportionally to the amount of co-ligand bound (Fig. 5). Correcting for the GTP-independent Met-tRNA\(_A\) binding of this preparation of IF-MP (1.8 pmol), the stoichiometry of the ternary complex is 1.8 pmol of \([\gamma-^{32}\text{P}]\text{GTP}}\) to 1.6 pmol of Met-tRNA\(_A\).

Having established optimal conditions for GTP and Met-tRNA\(_A\) binding to IF-MP, experiments were conducted with freshly prepared IF-MP to examine more carefully the combined kinetics of GTP and Met-tRNA\(_A\) binding to IF-MP. The time course of GTP and Met-tRNA\(_A\) binding are distinct (Fig. 6). At the earliest time point (10 s) GTP binding is maximal and quickly decays to a constant value; in the presence of Met-tRNA\(_A\), the plateau value was 1.8 to 2.3 pmol above the level observed in the absence of Met-tRNA\(_A\). In contrast, at the earliest time point (10 s) the level of Met-tRNA\(_A\) bound to IF MP was minimal. Met tRNA\(_A\) binding quickly reached a plateau value (3.5 pmol) in the presence of GTP, while in the absence of GTP the binding of Met-tRNA\(_A\) remained linear for the length of the assay. The amount of GTP-dependent Met-tRNA\(_A\) bound to IF-MP at 3 min was about 2.6 pmol. This level of GTP-dependent Met-tRNA\(_A\) binding is slightly greater than the level of Met-tRNA\(_A\)-dependent GTP binding (2.1 pmol), but again is suggestive of a 1:1 stoichiometry.

Several other observations can be made from these data (Fig. 6): (a) Met-tRNA\(_A\) binding is stimulated by GTP, but a significant amount of Met-tRNA\(_A\) binding occurs in the absence of GTP; (b) in the absence of Met-tRNA\(_A\), \(\alpha\)- and \(\gamma\)-labeled GTP do not form a stable complex with IF-MP; (c) \([^{14}\text{C}]\text{GTP}}\) binds to a greater extent than either \([\alpha-^{32}\text{P}]\text{GTP}}\) or \([\gamma-^{32}\text{P}]\text{GTP}}\). Since there is no hydrolysis of GTP under the conditions of complex formation (data not shown), all three forms of GTP should bind in the same manner. Therefore, it appears that \([^{14}\text{C}]\text{GTP}}\) gives an artificially high value; however, we have not been able to verify this experimentally.

Inactivation of IF-MP—Although IF-MP can bind Met-tRNA\(_A\) by GTP-dependent and GTP-independent mechanisms, stable GTP binding by IF-MP is strictly Met-tRNA\(_A\)-dependent. As shown in Fig. 7, preincubation of IF-MP with GTP in the absence of Met-tRNA\(_A\) rapidly inactivates the ability of IF-MP to form a ternary complex upon subsequent addition of Met-tRNA\(_A\). To determine whether such inactivation results from decreased formation of the ternary complex or retention of the complex to nitrocellulose filters, or both, IF-MP was preincubated with GTP in the presence or absence of 40 S ribosomal subunits prepared from salt-washed polysomes (Table II). If IF-MP is preincubated with GTP in the absence of 40 S subunits, inactivation of GTP-dependent Met-tRNA\(_A\) binding is obtained with subsequent binding conducted in the presence or absence of 40 S subunits (Table II, 2 and 5). If IF-MP is first preincubated with 40 S subunits, addition of GTP prior to the addition of Met-tRNA\(_A\) does not produce inactivation (Table II, 6). These data indicate that preincubation of IF-MP with GTP inactivates both ternary complex formation and binding. In agreement with other reports, IF-MP binding activity is also inactivated by 0.1 mM aurintricarboxylic acid and 1 mM N-ethylmaleimide (Table II, 7 and 8), but not by 1 mM fusidic acid (data not shown).

Binding of IF-MP-Met-tRNA\(_A\) Complexes to 40 S Ribosomal Subunits—Since IF-MP can form stable binary (IF-MP-Met-tRNA\(_A\)) or ternary (IF-MP-GTP-Met-tRNA\(_A\)) complexes, binding of each of these complexes to 40 S ribosomal subunits was examined. Following formation of these complexes as described earlier, aliquots were directly analyzed on nitrocellulose filters to determine the amount of complex formed; 40 S ribosomal subunits were then added to the remainder to determine the extent of complex binding to these. Binding of

---

**Fig. 4.** Double reciprocal plots of GTP-dependent Met-tRNA\(_A\) binding to IF-MP as a function of Met-tRNA\(_A\) and GTP concentrations. Results obtained with highly purified rat liver \([^{14}\text{C}]\text{Met-tRNA}}\) (O-O), 460 cpm/pmol, and partially purified rabbit liver \([^{35}\text{S}]\text{Met-tRNA}}\) (O-O), 12,000 cpm/pmol, are compared. A, initial velocity of Met-tRNA\(_A\) binding as a function of Met-tRNA\(_A\) concentration by 1.7 mg of IF-MP; the concentration of GTP was 10 \(\mu\text{M}\). B, initial velocity of Met-tRNA\(_A\) binding by 1.7 \(\mu\text{g}\) of IF-MP as a function of GTP concentration; 25 pmol of Met-tRNA\(_A\) were used per 50 \(\mu\text{l}\) of reaction. The rate of GTP-independent Met-tRNA\(_A\) binding was subtracted from the rate of total Met-tRNA\(_A\) binding measured at 15 s.

**Fig. 5.** Extent of GTP and Met-tRNA\(_A\) binding as a function of Met-tRNA\(_A\) concentration. IF-MP (1.7 \(\mu\text{g}\)) was incubated with 10 \(\mu\text{M}\) \([\gamma-^{32}\text{P}]\text{GTP}}\) (8,000 cpm/pmol) for 5 min at 0° in the presence of the indicated amount of \([^{14}\text{C}]\text{Met-tRNA}}\) (460 cpm/pmol). The preparation of IF-MP used had been stored frozen in liquid \(N\) for 1 week prior to use. In the absence of added Met-tRNA\(_A\), 0.2 pmol of \([\gamma-^{32}\text{P}]\text{GTP}}\) were bound. In the absence of GTP, 1.8 pmol of \([^{14}\text{C}]\text{Met-tRNA}}\) were bound when 20 pmol of \([^{14}\text{C}]\text{Met-tRNA}}\) were added to the reaction mixture. No background values have been subtracted.
MINUTES

FIG. 7. Inactivation of GTP-dependent Met-tRNA<sub>f</sub> binding by preincubation with GTP. IF-MP (1.7 μg) was incubated with 10 μM GTP under standard binding conditions for the indicated times. Subsequently 25 pmol of [³H]Met-tRNA<sub>f</sub> (88 cpm/pmol) were added and incubation carried out for an additional 10 min; -- -- --, the level of GTP-independent binding of Met-tRNA<sub>f</sub> by IF-MP not preincubated with GTP.

these complexes to 40 S ribosomal subunits was then analyzed by sucrose gradient analysis of formaldehyde fixed samples (19). In the presence of GTP, 5 pmol of ternary complex are formed and 0.45 pmol of this are bound to 40 S ribosomal subunits (Fig. 8B). The 40 S subunit complex contains equal molar quantities of Met-tRNA<sub>f</sub> and GTP. Under the conditions used only 10% of the ternary complex is recovered in the 40 S region. In the absence of GTP, 3.3 pmol of binary complex are formed and 0.3 pmol is bound to 40 S subunits (Fig. 8C). Bound Met-tRNA<sub>f</sub> as assayed on nitrocellulose filters appears to be transferred with equal efficiency (approximately 10%) to 40 S ribosomal subunits whether initially present as ternary complexes with GTP or as a binary complex. Subsequent addition of GTP to IF-MP incubated with Met-tRNA<sub>f</sub> results in additional binding of 0.12 pmol of Met-tRNA<sub>f</sub> to 40 S subunits with the total bound, 0.42 pmol, comparable to that obtained with ternary complex formation (Fig. 8D). However, GTP is incorporated only to the extent that additional Met-tRNA<sub>f</sub> is bound, 0.13 pmol (Fig. 8D). Previously formed IF-MP-Met-tRNA<sub>f</sub> binary complex, therefore, does not appear to be converted to a ternary complex containing GTP. Such data would support the compulsory ordered mechanism and 1:1 stoichiometry of GTP and Met-tRNA<sub>f</sub> binding indicated by kinetic studies of ternary complex formation. It should be noted that no exogenous template (AUG or mRNA) was added in these studies. Addition of AUG to a level of 0.3 A<sub>254</sub> unit per 50 μl of reaction mixture shows no additional stimulation of 40 S complex formation as assayed by sucrose gradients (data not shown).

DISCUSSION

IF-MP, homogeneous by criteria presented in the accompanying paper (24), rapidly forms a stable complex with the initiator Met-tRNA<sub>f</sub> (and to some extent Met-tRNA<sub>a</sub> also), but not other aminoacyl-tRNA species (Table I). The ternary complex IF-MP-GTP-Met-tRNA<sub>f</sub> binds to nitrocellulose filters in the absence of ribosomal subunits. Under optimal assay conditions 5 pmol of Met-tRNA<sub>f</sub> and 4 pmol of GTP are bound per μg of IF-MP (Fig. 3B). Based on a molecular weight of

FIG. 6. Time course of GTP-dependent and GTP-independent Met-tRNA<sub>f</sub> binding, and Met-tRNA<sub>a</sub>-dependent and Met-tRNA<sub>f</sub>-independent GTP binding, by IF-MP. IF-MP (1.7 μg) was incubated under standard binding conditions in 50 μl containing 20 pmol of [³H]Met-tRNA<sub>f</sub> (460 cpm/pmol) or 10 μM GTP, or both; A, [³H]GTP, 9,000 cpm/pmol; B, [³H]GTP, 12,000 cpm/pmol; C, [³H]GTP, 9,000 cpm/pmol. Binding was started by the addition of IF-MP to the reaction mixture. No correction for background was made.
utilized by other laboratories in several important aspects. In tRNA, binding by IF-MP differ significantly from those complex formation appear elsewhere (13, 32).

and the physical properties discussed in the preceding paper in methionyl-puromycin formation. Based on these activities 40 S ribosomal subunit initiation complex; and (c) participate Met-tRNA, to nitrocellulose filters; (b) bind Met-tRNA, to the fractions which form stable ternary complexes with Met-tRNA, in the presence of GTP. These fractions also: (a) bind tRNA,; or (d) increased binding might result from use of populations of IF-MP exist which bind either GTP or Met-tRNA, achieved under these assay conditions; (c) two functional possibilities are: (a) only 50% of the IF-MP preparation used in the active species, a 1:l:l ratio would be obtained. Four other 90,000 (see Ref. 24) for the IF-MP monomer, a 2 IF-MP/l stoichiometry is indicated. However, data presented in the preceding paper (24) show that IF-MP may also exist as a stable 180,000 molecular weight dimer. If this is the active species, a 1:1:1 ratio would be obtained. Four other possibilities are: (a) only 50% of the IF-MP preparation used in these experiments is active; (b) maximal binding is not achieved under these assay conditions; (c) two functional populations of IF-MP exist which bind either GTP or Met-tRNA,; or (d) increased binding might result from use of homogeneous Met-tRNA,.

Several laboratories (9-18) have partially purified protein fractions which form stable ternary complexes with Met-tRNA, in the presence of GTP. These fractions also: (a) bind Met-tRNA, to nitrocellulose filters; (b) bind Met-tRNA, to the 40 S ribosomal subunit initiation complex; and (c) participate in methionyl-puromycin formation. Based on these activities and the physical properties discussed in the preceding paper (24), these preparations appear to be similar to IF-MP. Detailed studies of IF-MP activity other than Met-tRNA, complex formation appear elsewhere (13, 32).

Assay conditions established in the present paper for Met-tRNA, binding by IF-MP differ significantly from those utilized by other laboratories in several important aspects. In contrast to prolonged incubation at 25 or 37° (10, 22), homogeneous IF-MP maximally binds Met-tRNA, in the presence of GTP by 1 to 2 min at 0°. Initial rates of binding are obtained only at 15 s or less. Optimal GTP and Met-tRNA, concentrations for ternary complex formation with IF-MP are also 1 to 2 orders of magnitude lower than conditions used by other laboratories (9, 10, 12, 22). Cashion and Stanley (11) report that maximal binding with their IF-1 is obtained only when Met-tRNA, is present in a 1000-fold excess. In contrast, 1.7 µg of IF-MP (15 pmol of monomer) is saturated with 7.5 pmol of Met-tRNA, out of a total of 30 pmol (25% binding). Since GTP and Met-tRNA, are required for a maximal extent of binding, and the rate of ternary complex formation is dependent on the concentration of IF-MP, differences between homogeneous IF-MP and other functionally identical Met-tRNA, binding factors may reflect molecular heterogeneity or inhibitory components present in these latter preparations, or both.

The ratio of GTP-dependent to GTP-independent Met-tRNA, binding has been presented in several papers as evidence of extensive purification (9-12). Data presented here demonstrate that IF-MP can form stable complexes with Met-tRNA, in the presence or absence of GTP (Fig. 3). While freshly prepared IF-MP binding activity is stimulated 20-fold by GTP (rate, 15 s), this stimulation is reduced to about 2-fold if the incubation time is prolonged (extent, 15 min). In addition to time of incubation, other considerations such as Mg* concentration in the wash buffer, aging or freezing of IF-MP, purity of the Met-tRNA, and the extent of ligand saturation of IF-MP affect the GTP dependence as well as the extent of Met-tRNA, binding to IF-MP. Thus, while IF-MP-directed binding of Met-tRNA, is always stimulated by GTP, it is clear that the magnitude of this stimulation is sufficiently variable to warrant caution in correlating factor purity with GTP dependence.

The formation of stable binary and ternary complexes of IF-MP is summarized in Fig. 9. Ternary complex formation proceeds by the ordered addition of GTP and Met-tRNA, The subsequent interaction of ternary complex and 40 S subunits leads to the transfer of equal molar quantities of Met-tRNA, and GTP to the 40 S subunits. Since GTP and Met-tRNA, are required for a maximal extent of Met-tRNA, binding to IF-MP (Fig. 7), which occurs without GTP hydrolysis. This inactivation is prevented by the presence of 40 S subunits (Table II), suggesting the possibility that in vivo the first step in this pathway might be the binding of IF-MP to the 40 S subunit. The first step in the formation of a binary complex appears to be the conversion of IF-MP to IF-MP* (an altered state of the native molecule). This conversion is a slow process (Fig. 3B) as the binding activity of freshly prepared IF-MP displays a 20-fold stimulation by GTP (rate, Fig. 3A). However, under assay conditions (minus GTP) the conversion to IF-MP* is increased because after 5 min the level of Met-tRNA, binding is 50% of that in the presence of GTP. These data may indicate that an equilibrium exists between IF-MP and IF-MP*. The removal of IF-MP* by binary complex formation might then lead to the continued conversion of IF-MP to IF-MP* until a new equilibrium is reached between IF-MP, IF-MP*, and IF-MP*-Met-tRNA, Based on studies of (a) inactivation of IF-MP by GTP (Fig. 7), (b) binding of radioactive GTP to...
IF-MP (Fig. 5), and (c) 40 S-Met-tRNA\textsubscript{A} complex formation (Fig. 8), it seems likely that IF-MP\textsuperscript{*} does not interact with GTP either as free IF-MP\textsuperscript{*} or as a binary complex. The binary complex of IF-MP\textsuperscript{*} and Met-tRNA\textsubscript{A} is identical to the ternary complex IF-MP-GTP-Met-tRNA\textsubscript{A} in terms of effectiveness of transfer of Met-tRNA\textsubscript{A} to 40 S subunits (Fig. 8). The functional efficiency of the binary and ternary complexes in peptide bond formation is currently being investigated.

The above observations on the binary and ternary complex pathways are suggestive evidence for an allosteric role for GTP. Additional evidence for this is that at no time during the reaction is there any appreciable GTP hydrolysis, a fact supported by the ready substitution of GMP-P(CH\textsubscript{2})P for GTP (data not shown; see also Refs. 9 and 22). Attempts to study the function of GTP after the formation of a 40 S-Met-tRNA\textsubscript{A}-GTP complex have not yet been successful since one of the initiation factors (IF-M2A) required for 40 S complex formation is capable of hydrolyzing GTP in the presence of 40 S and 60 S subunits (33).

The homologous prokaryotic factor IF-2 also forms a stable ternary complex with initiator tRNA (fMet-tRNA\textsubscript{A}) which binds first to the small ribosomal subunit during initiation complex assembly (34-37). In contrast to IF-MP, 5 mM Mg\textsuperscript{2+} is required for ternary complex formation with IF-2 (38). Although no hydrolysis of GTP occurs during ternary complex formation, IF-2 does catalyze GTP hydrolysis in the presence of both ribosomal subunits and appears to promote joining of the 30 and 50 S ribosomal subunits (34, 38-40).

In addition to forming a ternary complex with Met-tRNA\textsubscript{A} and GTP, IF-MP prepared in this laboratory has been shown to: (a) catalyze methionyl-puromycin formation with both natural and artificial mRNAs (13, 24); (b) bind to (as a ternary complex) to the 40 S ribosomal subunit (see Refs. 32 and 41, and "Results"); (c) reverse hemin-deficient inhibition of translation (19, 20); (d) bind to poly(A), globin mRNA, R17 tRNA, and double-stranded RNA (18, 20); and (e) reverse inhibition of translation by double-stranded RNA and oxidized glutathione (41). Whether all of these activities are related to a common mechanism of action of IF-MP during establishment of the initiation complex is not known at present, but such observations may be relevant to the possible regulation of protein synthesis at the translational level.

Acknowledgments—We wish to thank Mr. Wayne M. Kemper for his assistance in the preparation of IF-MP and Ms. Rebecca Rawls for her technical assistance.

REFERENCES
1. Shafritz, D. A., and Anderson, W. F. (1970) Nature 227, 918-920
2. Merrick, W. C., and Anderson, W. F. (1975) J. Biol. Chem. 250, 1197-1206
3. Crystal, R. G., and Anderson, W. F. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 1086-111
4. Gastor, E., and Moldave, K. (1972) J. Mol. Biol. 66, 391-402
5. Leader, D. P., and Wool, I. G. (1972) Biochim. Biophys. Acta 262, 369-370
6. Zaslowski, M., and Ochoa, S. (1973) J. Mol. Biol. 73, 65-76
7. Eich, F., and Drews, J. (1974) Biochim. Biophys. Acta 340, 334-338
8. Grummt, F. (1973) Eur. J. Biochem. 43, 337-342
9. Levin, D. H., Kyner, D., and Arcy, G. (1973) Proc. Natl. Acad. Sci. U. S. A. 70, 41-45
10. Gupta, N. K., Woodley, C. L., Chen, Y. C., and Bose, K. K. (1973) J. Biol. Chem. 248, 4500-4511
11. Cashon, L. M., and Stanley, W. M., Jr. (1973) Biochim. Biophys. Acta 324, 410-419
12. Schreier, M. H., and Staehelin, T. (1973) Nature New Biol. 245, 1286-1290
13. Elson, N. A., Adams, S. C., Merrick, W. C., Safer, B., and Anderson, W. F. (1975) J. Biol. Chem. 250, 9074-9079
14. Dettman, G. L., and Stanley, W. M., Jr. (1972) Biochim. Biophys. Acta 287, 124-135
15. Chen, C. Y., Woodley, C. L., Bose, K. K., and Gupta, N. K. (1972) J. Biol. Chem. 247, 1-9
16. Levin, D. H., Kyner, D., and Acy, G. (1973) J. Biol. Chem. 248, 6416-6425
17. Merrick, W. C., Safer, B., Adams, S., and Kemper, W. (1974) Fed. Proc. 33, 1269
18. Hellingman, J. A., and Shafritz, D. A. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 1021-1025
19. Clemens, M. J., Henschaw, E. C., Rahaminoff, H., and London, I. M. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 2946-2950
20. Kaempfer, R. (1974) Biochem. Biophys. Res. Commun. 61, 541-547
21. Schreier, M. H., and Staehelin, T. (1973) in Regulation of Transcription and Translation in Eukaryotes (Bautz, E. F. K., Karlson, P., and Kersten, H., eds), Springer-Verlag, Berlin
22. Cashon, L. M., Dettman, G. L., and Stanley, W. M., Jr. (1974) Methods Enzymol. 30, 153-171
23. Gupta, N. K., Chatterjee, B., Chen, Y. C., and Majumdar, A. (1975) J. Biol. Chem. 250, 852-862
24. Safer, B., Anderson, W. F., and Merrick, W. C. (1975) J. Biol. Chem. 250, 9076-9075
25. Pearson, R. L., Weiss, J. F., and Kelmers, A. D. (1971) Biochim. Biophys. Acta 228, 770-774
26. Gilbert, J. M., and Anderson, W. F. (1970) J. Biol. Chem. 245, 2342-2349
27. Palley, A. K., Kantor, J. A., Robert-Guroff, M. G., Picierno, D. J., Weiss, G. B., Vavich, J. M., and Anderson, W. F. (1974) J. Biol. Chem. 249, 7049-7056
28. Crystal, R. G., Elson, N. A., and Anderson, W. F. (1974) Methods Enzymol. 30, 101-127
29. Clandon, W. W. (1963) Biochim. Biophys. Acta 67, 104-137
30. Clandon, W. W. (1963) Biochim. Biophys. Acta 67, 199-196
31. Rouget, P., and Chapelier, F. (1968) Eur. J. Biochem. 4, 305-309
32. Adams, S. L. (1975) Ph.D. thesis, George Washington University
33. Merrick, W. C., Kemper, W. M., and Anderson, W. F. (1975) J. Biol. Chem. 250, 5556-5562
34. Fukund, J. L., and Hershey, J. W. B. (1973) J. Biol. Chem. 248, 4206-4212
35. Rudland, P. S., Whvbrow, W. A., and Clark, B. F. (1971) Nature New Biol. 231, 76-78
36. Lockwood, A. H., Chakraborthy, P., and Mair, U. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 3122-3126
37. Remold, O. (1970) Trans. Thach, R. E. (1970) J. Biol. Chem. 245, 5737-5742
38. Grunberg-Manago, M., Godefroy-Colbum, T., Wolff, A. D., Deacon, P., Pantaloni, D., Springaa, M., Dondon, J., and Kay, A. (1973) in Regulation of Transcription and Translation in Eukaryotes (Bautz, E. F. K., Karlson, P., and Kersten, H., eds), Springer-Verlag, Berlin
39. Kolakofsky, D., Dewey, K. F., Hershey, J. W. B., and Thach, R. E. (1968) Proc. Natl. Acad. Sci. U. S. A. 61, 1066-1070
40. Leong, J. C., Grunberg-Manago, M., Dondon, J., Gros, D., and Gros, F. (1970) Nature 226, 505-510
41. Clemens, M. J., Safer, B., Merrick, W. C., Anderson, W. F., and London, I. M. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 1285-1290
Binding of MET-TRNAf and GTP to homogeneous initiation factor MP.
B Safer, S L Adams, W F Anderson and W C Merrick

J. Biol. Chem. 1975, 250:9076-9082.

Access the most updated version of this article at http://www.jbc.org/content/250/23/9076

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/250/23/9076.full.html#ref-list-1