*POLYPEPTIDE CHAIN INITIATION IN E. COLI: SULFHYDRYL GROUPS AND THE FUNCTION OF INITIATION FACTOR F_2**

By Rajarshi Mazumder, Yung-Bog Chae, and Severo Ochoa

DEPARTMENT OF BIOCHEMISTRY, NEW YORK UNIVERSITY SCHOOL OF MEDICINE

Communicated February 24, 1969

Abstract.—The activity of the chain initiation factor F_2 in promoting the messenger-dependent binding of formylmethionyl-transfer RNA (fMet~$tRNA_f$) to purified *E. coli* ribosomes is inhibited by sulfhydryl-binding reagents, such as N-ethylmaleimide or *p*-hydroxymercuribenzoate, but prior incubation with guanosine triphosphate (GTP) or with ribosomes largely prevents this inhibition. The effect of GTP suggests that it forms a complex with F_2 whereby "active" sulfhydryl groups become sheltered. Experiments on the time course of the binding reaction, with and without preincubation of F_2 with GTP, and gel filtration experiments with ^{3}H-labeled GTP lend support to this suggestion.

As shown in several laboratories, GTP is needed for formation of the chain initiation complex in *E. coli*, a reaction that requires the presence of the initiation factors F_1, F_2, and F_3.[1] In a previous paper[2] we reported on the isolation of electrophoretically homogeneous F_2 and its relation to the ribosomal proteins. In this communication we present experiments suggesting that the involvement of GTP in chain initiation is related to an interaction with F_2 with formation of a GTP-F_2 complex. Substantial protection by GTP from the inhibition of F_2 by sulfhydryl-binding reagents further suggests that "essential" sulfhydryl group of the protein become less accessible to these reagents as a result of the GTP-F_2 interaction.[3]

Materials and Methods.—The preparation of ribosomes, F_2, F_1, fraction S, F_2 assay, and the sources of various materials were as in the previous paper.[2] Experiments were performed with F_2 preparations of different specific activities, including homogeneous F_2 (step 6),[2] step 5 F_2, or with less active fractions. In every case, the specific activity is indicated in the table legends; 30*S* and 50*S* ribosomal subunits were prepared from purified *E. coli* Q13 ribosomes, as in previous work.[4] The subunits were concentrated without dialysis by high-speed centrifugation. Ribosomal subunits are usually less stable than unfractionated ribosomes and were utilized within 24 hr of preparation. GTP was obtained from PL Biochemicals, ^{3}H-labeled GTP from Schwarz BioResearch, Inc., and N-ethylmaleimide and *p*-hydroxymercuribenzoate from the Sigma Chemical Co. Sephadex G-50 (Pharmacia, fine, particle size 20–80 μ) was treated prior to use with 2.0 mM EDTA, and the EDTA was removed by thorough washing with water.

Experiments with sulfhydryl-binding reagents: Prior to assay, F_2 was preincubated, without or with N-ethylmaleimide or *p*-hydroxymercuribenzoate, in a buffer containing 20 mM Tris-HCl, pH 7.8, 20 mM NH_4Cl, 0.2 mM magnesium acetate, and 5% glycerol (v/v). Aliquots of each sample were then assayed for F_2 activity with the standard assay.[2] The dilution of preincubation samples for F_2 assay was from 10- to 20-fold. In GTP protection experiments, when N-ethylmaleimide was used, GTP was either preincubated simultaneously with F_2 and the reagent, or incubated with F_2 prior to reagent addition. When the inhibitor was *p*-hydroxymercuribenzoate, GTP was always incubated with F_2 before the reagent was added. Full details are given in the footnotes to the tables.

Interaction of GTP and F_2: The time course of the fMet~$tRNA_f$ binding reaction

was followed after preincubation without or with GTP. Experimental details are given in the legend to Table 4. Formation of a GTP-protein complex was studied with the technique described by Gordon[5] for isolation of the GTP-T factor-phe~tRNA complex, with use of gel filtration of Sephadex G-50. The reaction mixture contained (in a final volume of 0.05 ml) 100 mM Tris-HCl, pH 7.2, 100 mM NH_4Cl, 10 mM magnesium acetate, 2 mM dithiothreitol, 0.11 mM ^{3}H-GTP (specific radioactivity, 177 cpm/$\mu\mu$mole), and either (*a*) no further additions, (*b*) 4 μg (about 50 $\mu\mu$moles) of step 6 F_2 (specific activity, 16), (*c*) 4 μg (about 60 $\mu\mu$moles) of crystalline bovine plasma albumin, or (*d*) 4 μg F_2 previously incubated for 10 min at 25° with 1.0 mM *p*-hydroxymercuribenzoate in a buffer containing 20 mM Tris-HCl, pH 7.8, 20 mM NH_4Cl, 0.2 mM magnesium acetate, and 5% glycerol (v/v). After incubation for 7 min at 25° and cooling in ice, 0.05 ml of a buffer containing 100 mM Tris-HCl, pH 7.2, 100 mM NH_4Cl, 10 mM magnesium acetate, 2 mM dithiothreitol, and 50% glycerol (v/v) was added. In the *p*-hydroxymercuribenzoate experiment, dithiothreitol was omitted from all buffers. The mixture was immediately transferred to a Sephadex G-50 column (0.8 × 19 cm) and the column washed, at a flow rate of about 3 ml/hr, with the same buffer. The first milliliter was discarded and collection of fractions (0.2 ml) was then started. Gel filtration was carried out at 4°. Aliquots (0.15 ml) of each fraction were counted for 20 min, in 10 ml of Bray's solution, in a Packard Tri-Carb liquid scintillation spectrometer.

Results.—Inhibition of F_2 by sulfhydryl-binding agents: As is shown in Table 1, the activity of F_2 is inhibited by incubation with N-ethylmaleimide or *p*-hydroxymercuribenzoate prior to assay. *p*-Hydroxymercuribenzoate is a more potent inhibitor; at 10^{-4} *M* concentration it inactivates F_2 to about the same extent as a 100-fold higher concentration of N-ethylmaleimide. The F_2 assay samples contained ribosomes and other initiation factors, and it was important to rule out an effect on these components by the unreacted N-ethylmaleimide

TABLE 1. *Effect of sulfhydryl-binding reagents on F_2 activity.*

Expt. no.	Sample	Preincubation	Relative activity	Inhibition (%)
1*	(*a*)	F_2	100	0
	(*b*)	F_2 + NEM (10 mM)	24	76
	(*c*)	F_2 + NEM (1 mM)	44	56
2†	(*a*)	F_2	100	0
	(*b*)	F_2 + NEM (10 mM)	26	74
	(*c*)	F_2 + NEM (10 mM)	38	62
	(*d*)	F_2 + NEM (10 mM) + DTT (50 mM)	96	4
3‡	(*a*)	F_2	100	0
	(*b*)	F_2 + PMB (0.1 mM)	27	73
4§	(*a*)	F_2	100	0
	(*b*)	F_2 + NEM (20 mM)	31	69
	(*c*)	F_2 + NEM (10 mM)	40	60

After preincubation at 25° (30 min, unless otherwise stated), F_2 activity was immediately assayed at a suitable dilution. The absolute activities (fMet(^{14}C) ~tRNA bound, in $\mu\mu$moles/assay sample), in the absence of inhibitor (100% values), were: expt. 1 (F_2, original specific activity (spec. act.) 6.5, 0.3 μg), 1.95; expt. 2 (F_2, spec. act. 3, 1.5 μg), 3.6; expt. 3 (F_2, spec. act. 3, 1.5 μg), 2.5; expt. 4 (F_2, spec. act. 16, 0.19 μg), 1.6.

Abbreviations used in tables: NEM, N-ethylmaleimide; DTT, dithiothreitol; PMB, *p*-hydroxymercuribenzoate.

* After preincubation, samples were dialyzed for 4 hr at 4° against a buffer containing 20 mM Tris-HCl, pH 7.8, 20 mM NH_4Cl, 0.2 mM magnesium acetate, and 5% glycerol (v/v) to remove excess NEM.

† Samples not dialyzed after preincubation; NEM concentration in assay of sample (*b*), 1.0 mM; sample (*c*), excess NEM removed by adding DTT (final concentration, 50 mM) and incubating for a further 5 min before assay; sample (*d*), 50 mM DTT present throughout preincubation period.

‡ Preincubation, 5 min; samples not dialyzed; PMB concentration in assay, 0.01 mM.

§ Excess NEM removed by addition of DTT, as in expt. 2, sample (*c*).

or *p*-hydroxymercuribenzoate carried over with F_2 into the assay mixtures. It may be seen in Table 1 that removal of unreacted N-ethylmaleimide after preincubation, either by dialysis (expt. 1) or by addition of excess dithiothreitol (expt. 2, sample (*c*); expt. 4, samples (*b*) and (*c*)), does not affect the results (cf. expt. 2, sample (*b*)). It may further be seen (expt. 2, sample (*d*)) that excess dithiothreitol protects F_2 from inactivation by N-ethylmaleimide. As regards *p*-hydroxymercuribenzoate, its final concentration in the assay samples was 10^{-5} *M*. At this concentration this reagent does not significantly inhibit the fMet~tRNA ribosomal binding reaction. Preincubation of ribosomes with 10 mM N-ethylmaleimide, followed by the removal of unreacted compound, did not significantly affect their activity in the binding reaction. We conclude that the integrity of certain sulfhydryl groups is essential for F_2 activity.

Protection by GTP: The GTP analog 5′-guanilyldiphosphonate has been shown to replace GTP in the ribosomal binding of fMet~$tRNA_f$ but not in fMet-puromycin synthesis; this suggests that the function of GTP in formation of the chain initiation complex may be of a conformational nature.[1] The results to be reported in this section suggest that F_2 may provide a site(s) for such role of GTP. As may be seen in Table 2, at 2×10^{-3} *M* concentration GTP protects F_2 against inactivation by N-ethylmaleimide (expts. 1 and 4) or *p*-hydroxymercuribenzoate (expt. 3) almost completely. It also afforded substantial protection against N-ethylmaleimide inactivation at 2×10^{-4} *M* (expt. 2). At this concentration ATP, CTP, or UTP did not protect. This indicates that the effect of GTP is specific for this ribonucleoside triphosphate. It should be added that (crude) fMet~tRNA did not increase the protective effect of GTP.

TABLE 2. *Protection by GTP from F_2 inactivation by sulfhydryl-binding reagents.*

Expt. no.	Preincubation	Relative activity	Inhibition (%)
1	F_2	100	0
	F_2 + NEM (10 mM)	22	78
	F_2 + GTP (2 mM) + NEM	95	5
2	F_2	100	0
	F_2 + NEM (10 mM)	36	64
	F_2 + GTP (0.2 mM) + NEM	75	25
	F_2 + ATP (0.2 mM) + NEM	43	57
	F_2 + CTP (0.2 mM) + NEM	41	59
	F_2 + UTP (0.2 mM) + NEM	36	64
3	F_2	100	0
	F_2 + PMB (0.1 mM)	26	74
	F_2 + GTP (2 mM), then PMB	81	19
4	F_2	100	0
	F_2 + NEM (10 mM)	49	51
	F_2 + GTP (2 mM) then NEM	94	6

In expts. 1 and 2, F_2 was preincubated for 30 min at 25° either alone, with NEM, or with NEM and ribonucleoside triphosphates, and assayed, either immediately after suitable dilution (expt. 1) or following removal of excess NEM, by addition of 50 mM DTT and incubation for a further 5 min (expt. 2). In expt. 3, F_2 was preincubated at 25°, either alone for 7 min or with GTP (2 min) followed by PMB (5 min), and assayed immediately after suitable dilution. In expt. 4, F_2 was preincubated at 25°, either alone (35 min), with NEM (35 min), or with GTP (2 min) followed by NEM (33 min). Excess NEM was removed with DTT as in expt. 2. The absolute activities (expressed as in Table 1), in the absence of inhibitor (100% values), were: expt. 1 (F_2, spec. act. 3, 1.5 μg), 2.8; expt. 2 (F_2, spec. act. 3, 1.5 μg), 3.2; expt. 3 (F_2, spec. act. 3, 1.5 μg), 2.2; expt. 4 (F_2, spec. act. 16, 0.25 μg), 2.5. Other conditions as in Table 1.

Protection by ribosomes: We found that 70*S* ribosomes also protect F_2 from inactivation by sulfhydryl-binding reagents. Typical results are shown in Table 3 (expts. 1, 3, and 4). When F_2 was preincubated with N-ethylmaleimide in the presence of relatively low concentrations of ribosomal subunits (expt. 2), there was significant protection of F_2 activity by the 30*S* but not by the 50*S* ribosomes. These results, which suggest the masking of essential sulfhydryl groups of F_2 by binding to 30*S* ribosomes, are in line with the observation[6] that the initiation factors are derived from the 30*S* subunit.

Interaction between GTP and F_2: The protection by GTP against inactivation of F_2 by sulfhydryl-binding agents suggested that GTP might interact with F_2 to form a complex whereby essential sulfhydryl groups of the protein become sheltered. Two kinds of experiments lend support to this suggestion.

TABLE 3. *Protection by ribosomes from F_2 inactivation by sulfhydryl-binding reagents.*

Expt. no.	Preincubation	Relative activity	Inhibition (%)
1	F_2	100	0
	F_2 + NEM (10 mM)	39	61
	F_2 + 70*S* ribosomes (90 A_{260} units/ml) + NEM	100	0
2	F_2	100	0
	F_2 + NEM (10 mM)	29	71
	F_2 + 50*S* ribosomes (10 A_{260} units/ml) + NEM	26	74
	F_2 + 30*S* ribosomes (4 A_{260} units/ml) + NEM	50	50
3	F_2	100	0
	F_2 + PMB (0.1 mM)	33	67
	F_2 + 70*S* ribosomes (84 A_{260} units/ml), then PMB	73	27
4	F_2	100	0
	F_2 + NEM (10 mM)	38	62
	F_2 + 70*S* ribosomes (100 A_{260} units/ml), then NEM	68	32

Conditions for each experiment as in the corresponding ones of Table 2, except for the substitution of ribosomes for ribonucleoside triphosphates. The absolute activities/assay sample, in the absence of inhibitor (100% values), were: expt. 1 (F_2, spec. act. 3, 1.5 μg), 2.1; expt. 2 (F_2, spec. act. 3, 1.5 μg), 1.7; expt. 3 (F_2, spec. act. 3, 1.5 μg), 2.0; expt. 4 (F_2, spec. act. 16, 0.19 μg), 1.3. The ribosomes used for expt. 3 were dialyzed for 2 hr against 0.25 *M* NH_4Cl, 5 mM Mg^{++}, and 20 mM Tris-HCl, pH 7.8, to remove DTT.

(1) When the factor is incubated with GTP prior to assay of F_2 activity, the early rate of ribosomal binding of fMet~$tRNA_f$ is faster than when GTP is added at zero time. A typical experiment is shown in Table 4.

(2) The formation of a GTP-protein complex on incubation of F_2 with ^3H-labeled GTP can be shown by gel filtration of Sephadex G-50 as illustrated in Figure 1. For a molecular weight of 80,000,[2] 4 μg of step 6 F_2 used in this experiment (see *Materials and Methods*) correspond to 50 $\mu\mu$moles of F_2. The amount of GTP bound was 0.5 $\mu\mu$mole or 1 per cent of the F_2. Although the amount of complex detected under these conditions is quite small, it may be seen in Figure 1 that no GTP radioactivity is excluded in the absence of protein or when an equivalent amount of serum albumin (60 $\mu\mu$moles) is substituted for F_2. Figure 1 also shows that *p*-hydroxymercuribenzoate (1.0 mM) markedly decreases complex formation in the presence of F_2.

TABLE 4. *Effect of preincubation of F_2 with GTP on the rate of AUG-dependent binding of fMet~tRNA$_f$ to ribosomes.*

Reaction time (min)	fMet(^{14}C)~tRNA bound after Preincubation of: F_2 alone (*a*)	F_2 + GTP (*b*)	$\frac{(b)-(a)}{(a)} \times 100$
0.5	0.27	0.45	66
1.5	0.57	0.68	19
3.5	0.93	1.00	8
10.5	1.71	1.60	0

Standard F_2 activity assays were carried out, for the times indicated, after (preincubation (7 mine, at 25°, in a buffer containing 20 mM Tris-HCl, pH 7.8, 20 mM NH_4Cl. 0.2 mM magnesium acetate, 2 mM DTT, and 5% glycerol (v/v)) of F_2 alone or F_2 + GTP (2 mM). For assay, the Mg^{++} concentration was made 3.5 mM. Results expressed as net $\mu\mu$moles fMet(^{14}C) ~tRNA bound after subtraction of the blank without F_2. Concentration of F_2 (spec. act. 10) in preincubation medium, 50 μg/ml; in assay samples, 0.25 μg/0.05 ml.

fMet~tRNA or F_1 did not significantly increase complex formation under the conditions of our experiments. As we previously mentioned,[2] step 6 F_2 is contaminated with a small amount of elongation factor G and, as a result, exhibits some GTPase activity. Experiments similar to that of Figure 1, but with a mixture of ^{3}H-labeled and γ-^{32}P-labeled GTP, showed that some of the GTP in the complex was dephosphorylated.

Gel filtration (Sephadex G-200) of F_2 in the absence and presence of GTP showed no change in the elution pattern of F_2 activity. This indicates that interaction between GTP and F_2 does not lead to polymerization or depolymerization of the factor.

Discussion.—The experiments reported in this paper suggest that the initiation factor F_2 possesses sulfhydryl groups which are essential for its activity. Furthermore, GTP specifically protects F_2 against inactivation by sulfhydryl-binding reagents. This may be due to steric hindrance or to the occurrence of a conformational change upon formation of a GTP-F_2 complex, whereby the "active" sulfhydryl groups become masked. Formation of small amounts of a GTP-protein complex could actually be shown by gel filtration. The reasons for the detection of only small amounts of an apparently stable complex are not clear and we cannot rule out possibility that a protein contaminant, present in the F_2 preparation to the extent of about 1 per cent, is responsible for the observed results. Nevertheless, elongation factor G, which as previously

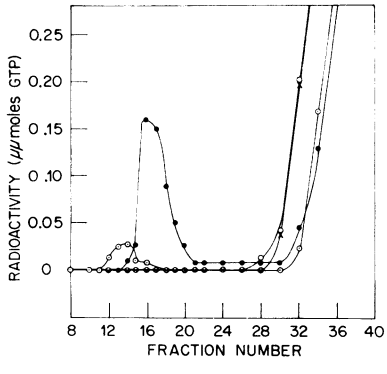

FIG. 1.—Isolation of (^{3}H)GTP-F_2 complex by gel filtration on Sephadex G-50. For details see *Materials and Methods.* ○—○, GTP alone; ●—●, GTP and F_2 (the amount of GTP eluted in fractions 14 through 21 corresponds to 0.5 $\mu\mu$mole); x—x, GTP and bovine plasma albumin; ⊙—⊙, GTP and F_2 previously incubated with PMB (the amount of GTP eluted in fractions 11 through 16 corresponds to 0.08 $\mu\mu$mole). F_2 was in all cases the step 6 preparation.

reported[2] contaminates step 6 F_2 to an extent of about 0.6 per cent, cannot be responsible because, as shown by Gordon,[5] it does not bind GTP and because factor T, which does bind the nucleotide,[5] was not detectable in 1.9 μg of step 6 F_2.[2] The observed inhibition of complex formation by *p*-hydroxymercuribenzoate in the presence of F_2, while suggestive of the involvement of F_2 itself, does not exclude the possibility that *p*-hydroxymercuribenzoate may inhibit formation of a complex between GTP and a contaminating protein.

The interactions between GTP and F_2, revealed by our work suggested functional analogies between this factor and elongation factor T which binds equimolar amounts of GTP and phe~tRNA.[5] However, we found no stimulation by fMet~tRNA of GTP binding in the presence of F_2, whereas the binding of GTP by factor T is strongly stimulated by phe~tRNA.[5] It may be remembered in this connection that, whereas the bulk of T is in the supernatant, F_2 along with other initiation factors is normally ribosome-bound. Thus, the interactions of F_2 reported here, while revealing interesting features of this protein, fall short of disclosing its mode of action in chain initiation. It seems reasonable to assume, however, that the requirement of GTP for chain initiation is related to these interactions.

We are indebted to Miss Roswitha Pagel for excellent technical assistance and to Miss Martha J. Miller for preparations of ribosomes and ribosomal subunits.

* Aided by grants AM-01845, FR-05099, and GM-01234 from the National Institutes of Health, USPHS.

[1] For references see Ochoa, S., *Naturwissenschaften*, **55**, 505 (1968).

[2] Chae, Y-B., R. Mazumder, and S. Ochoa, these PROCEEDINGS, **62**, 1181 (1969).

[3] Preliminary report: Mazumder, R., Y-B. Chae, and S. Ochoa, *Federation Proc.*, **28**, 597 (1969).

[4] Hille, M. B., M. J. Miller, K. Iwasaki, and A. J. Wahba, these PROCEEDINGS, **58**, 1652 (1967).

[5] Gordon, J., these PROCEEDINGS, **58**, 1574 (1967).

[6] Miller, M. J., and M. Zasloff, *Federation Proc.*, **28**, 880 (1969).