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Properties and Function of the Sulfhydryl Group in the Polypeptide Chain Elongation Factor G from *E. coli*

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The properties and function of sulfhydryl groups in the polypeptide chain elongation factor G (EF-G) have been studied by titration with *p*-chloromercuri[^{14}C]benzoate, or 5,5′-dithiobis(2-nitrobenzoic acid), and by assessing the inhibitory effect of N-ethylmaleimide on its activity. EF-G contained three sulfhydryl groups per mole of protein, of which only one was reactive under native conditions. The other two were nonreactive and could be titrated only after complete denaturation of the protein. The activity to catalyze the uncoupled GTPase reaction as well as the ability to form a ternary complex involving EF-G, guanine nucleotides, and ribosomes was completely inactivated by treatment of EF-G with N-ethylmaleimide.

The reactive sulfhydryl group was not required for interaction with guanine nucleotides, but was essential for binding to ribosomes. The binary complex involving EF-G and GDP could be isolated after treatment of EF-G with sulfhydryl reagents. The sulfhydryl group of EF-G was not protected by guanine nucleotides alone, but a marked protection was afforded by addition of ribosomes and GMP-P(CH_2)P.

The reactivity of the sulfhydryl group of EF-G was modulated by interaction with guanine nucleotides. The kinetic studies indicated that reactivity toward N-(1-anilinonaphthyl-4)-maleimide was reduced by GDP and, to a lesser extent, by GTP.

These results, together with the previous report on the spin-label probes (Arai, N., Arai, K., Maeda, T., Ohnishi, S., and Kaziro, Y. (1976) *J. Biochem.* **80**, 1057–1065) indicate that (1) the reactive sulfhydryl group of EF-G is required for interaction with ribosomes, and (2) its reactivity is modulated by guanine nucleotide-induced conformational transitions occurring near the reactive protein sulfhydryl.

Abbreviations: EF-G, prokaryotic elongation factor G; EF-2, eukaryotic elongation factor 2; GMP-P(CH_2)P, guanyl-5′-yl-methylenediphosphonate; GMP-P(NH)P, guanyl-5′-yl-imidodiphosphate; pCMB, *p*-chloromercuribenzoate; NEM, N-ethylmaleimide; ANS, 1-anilino-8-naphthalenesulfonic acid; ANM, N-(1-anilinonaphthyl-4)-maleimide; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); NTB, 2-nitro-5-thiobenzoate; and Na-DodSO_4, sodium dodecyl sulfate.

FF-G catalyzes a GTP-dependent translocation of a peptidyl-tRNA·mRNA complex from the aminoacyl- to the peptidyl-site on ribosomes with a concomitant release of a deacylated tRNA from the P site (see Ref. *1* for a review). It was found that the activity to hydrolyze GTP could be observed with EF-G and uncomplexed ribosomes, *i.e.*, in the absence of mRNA and tRNA derivatives (*2*, *3*). This activity, which is not coupled with translocation, was designated as an "uncoupled GTPase" (*4*) and studied as a simplified model system to understand the mechanism of EF-G-catalyzed reactions.

As described in our previous papers (*4–8*), the uncoupled GTPase reaction was shown to occur through the intermediary formation of a binary EF-G·GTP complex (*6–8*) and a ternary EF-G·GTP·ribosome complex (*4*, *5*). The latter complex was converted to the EF-G·GDP·ribosome complex which dissociated to yield an EF-G·GDP complex and ribosomes (*6*). Evidence for the existence of the EF-G·guanine nucleotide complex was based on the GTP- or GDP-induced change of fluorescence emission of an EF-G·ANS complex (*6*), and the differential ESR spectra of EF-G modified with spin-labeled sulfhydryl reagents in the presence of GTP or GDP (*7*). The direct isolation of the binary EF-G·guanine nucleotide complex was achieved by gel filtration (*6*) and by equilibrium dialysis (*8*). The formation of the ternary complex was reported by other investigators (*9–12*), and more recently, the binary complex formation was observed by Baca *et al.* (*13*) and Rohrbach and Bodley (*14*), using physical techniques and also from the kinetical studies.

The activity of EF-G is dependent on the presence of free sulfhydryl groups and the uncoupled GTPase reaction was strongly inhibited by several sulfhydryl reagents (*4*, *15*, *16*). By pretreatment of EF-G with iodoacetamide or NEM, it was shown that sulfhydryl group(s) in EF-G is essential for its activity (*4*). More recently, Rohrbach and Bodley (*17*) reported that the chemical modification of one cysteine residue of EF-G leads to the irreversible inactivation of its nucleotide binding activity. Their results are not consistent with ours, since we have demonstrated that modification of the one and only reactive sulfhydryl group of EF-G with NEM or its derivatives did not inhibit the formation of the binary EF-G·guanine nucleotides complexes (*6*, *7*).

The present paper describes further studies on the properties and function of the sulfhydryl group of EF-G which clearly demonstrate that the sulfhydryl group is not required for interaction with guanine nucleotides, but is required for binding of the EF-G·guanine nucleotide complex to ribosomes.

EXPERIMENTAL PROCEDURES

Materials—Crystalline EF-G was prepared from *E. coli* Q13 and MRE 600 cells according to the procedure previously described (*16*) with several modifications (*8*). [^{3}H]GDP and [^{3}H]GMP-P(CH_2)P were purchased from the Radiochemical Centre, Amersham, and purified by Dowex 1 column chromatography. [γ-^{32}P]GTP was prepared according to Glynn and Chappell (*18*) and purified by Dowex 1 column chromatography. [^{14}C]pCMB (specific activity 12.3 mCi/mmol) was obtained from the Commissariat à l'Energie Atomique, France and was purified by the method of Boyer (*19*). HEM and DTNB were purchased from Nakarai Chemicals, Kyoto, and Wako Pure Chemicals Industries, Osaka, respectively.

Titration of Sulfhydryl Groups—The titration of sulfhydryl groups in EF-G was carried out using DTNB according to Ellman (*20*) with some modifications (*21*) and using [^{14}C]pCMB by the method of Krakow and Goolsby (*22*). For determination of total thiol groups, EF-G was denatured with 6 M urea and 1% Na-DodSO_4. Urea solution was acidified to pH 2 with a concentrated HCl solution and kept at room temperature for 2 h to decompose cyanate, and then neutralized to pH 7.5 with NaOH immediately before use. For determination of reactive classes of sulfhydryl groups, the protein was titrated under native conditions in the presence or absence of guanine nucleotides as indicated.

Assay for EF-G Activity—Uncoupled GTPase reaction was carried out as described previously (*23*). The ability to form a ternary EF-G·ribosome·guanine nucleotide complex was determined by the previously published method (*23*) except that the concentration of NH_4Cl was 150 mM and the reaction was performed with a limiting amount of EF-G. When indicated, GTP was replaced by its nonhydrolyzable analogues, GMP-P(NH)P or

GMP-P(CH$_2$)P.

Formation of EF-G·guanine Nucleotide Complexes—The formation of the binary EF-G·guanine nucleotide complex was measured by equilibrium gel filtration or equilibrium dialysis, essentially as described previously (*6*, *8*), except for the use of the EF-G modified with NEM or pCMB.

(a) *Gel filtration of NEM-modified EF-G*: The crystalline EF-G (2.5 mg) was dissolved in 90 μl of a buffer containing 20 mM Tris-HCl (pH 7.6), 10 mM magnesium acetate and 36 μM GDP, and incubated with 5 μl of 0.15 M NEM at 0°C for 30 min. After 15 and 30 min, 1-μl aliquots were withdrawn, diluted 600-fold with a buffer solution containing 20 mM Tris-HCl (pH 7.6), 5 mM 2-mercaptoethanol, and assayed for the remaining GTPase activity. The NEM-treated EF-G was applied to a Sephadex G-25 column (0.54 × 52 cm) equilibrated with 20 mM Tris-HCl (pH 7.6), 10 mM magnesium acetate, and 36 μM [^{3}H]GDP (specific activity 4.2 μCi/μmol). The column was developed at 5°C with the above buffer and 0.28-ml fractions were collected and assayed for radioactivity, protein concentration, and GTPase activity.

(b) *Equilibrium dialysis of pCMB-modified EF-G*: The dialysis was performed in the same apparatus as described in a previous paper (*8*). The buffer solution (0.04 ml) contained 20 mM Tris-HCl (pH 7.6), 10 mM magnesium acetate, 0.1 M NH_4Cl, and 0.04 to 1 mM [^{3}H]GDP (specific activity 10 μCi/μmol). One side of the dialysis chamber contained the pCMB-modified EF-G at the concentration of 14 mg/ml. The pCMB-modified EF-G was prepared as follows. A solution containing EF-G (15 mg/ml) was treated with 0.83 mM of pCMB for 30 min at 0°C, and then dialyzed against above buffer (-GDP) for 3 h. Equilibrium dialysis was performed at 5°C and after 44 h, 20-μl aliquots were withdrawn and their radioactivity was determined.

Protection of EF-G from NEM Inactivation—The reaction mixture (50 μl) for NEM treatment contained 50 mM Tris-HCl (pH 7.6), 150 mM NH_4Cl, 10 mM magnesium acetate, 10 μM NEM, and 2.0 μg of EF-G, and, when indicated, 0.2 mM GTP, 10 μM GDP, or 10 μM GMP-P(CH$_2$)P, and 2.0 A_{260} units of ribosomes. The incubation was carried out at 0°C for 30 min, and stopped by the addition of 5 μl of 0.2 M 2-mercaptoethanol. After complementing the reaction mixture with other components (final volume, 75 μl) 5-μl aliquots were taken and assayed for the remaining uncoupled GTPase activity.

Measurements of the Reaction between EF-G and ANM by Fluorometric Procedure—The reaction between ANM and EF-G was followed fluorometrically in a microcuvette at 20°C using a Hitachi-Perkin Elmer MPF-2A fluorescence spectrophotometer. The reaction mixture (0.4 ml) contained 20 mM Tris-HCl (pH 7.9), 10 mM magnesium acetate, 2.0 nmoles of ANM, 0.16 mg (1.9 nmol) of EF-G, and 0.1 mM GTP, 0.1 mM GDP, or 1 mM GMP-P(NH)P as indicated. The details for the fluorescence measurement were as described previously (*8*, *24*).

Measurement of Protein Concentration—The protein concentration of EF-G was determined from absorbance at 280 nm (*25*) or 225 nm (*26*). $E^{1\%}_{1\,cm}$ values at 280 nm and 225 nm were 7.1 and 78.5, respectively, for purified EF-G. Taking the molecular weight of EF-G as 83,000, 1 μg of protein corresponds to approximately 12 pmol.

RESULTS

Titration of Sulfhydryl Groups in EF-G—Figure 1 shows the titration of sulfhydryl groups in EF-G with DTNB and [^{14}C]pCMB under various conditions. As shown in the figure, three sulfhydryl groups per mole of EF-G were titrated when the protein was denatured with 6 M urea or 6 M urea and 1% Na-DodSO$_4$. On the other hand, only one sulfhydryl group was titrated, when EF-G was incubated with DTNB or [^{14}C]pCMB under native conditions. The reaction of this sulfhydryl group with DTNB was very rapid and the reaction was complete within 1 min at 20°C (Fig. 1A). The reaction with [^{14}C]pCMB proceeded even faster and was complete within 1 min at 0°C (Fig. 1B). The effect of GDP or GTP on the reaction was also examined, and it was shown that these ligands did not protect the sulfhydryl group from DTNB or [^{14}C]pCMB titration.

Inactivation of EF-G Activity by NEM—The effect of pretreatment of EF-G with NEM on the ribosome-dependent GTPase activity and on the formation of a ternary EF-G·GMP-P(CH$_2$)P·ribosome complex was studied. As shown in Fig. 2, both activities decreased in parallel by treatment with relatively low concentration of NEM. About

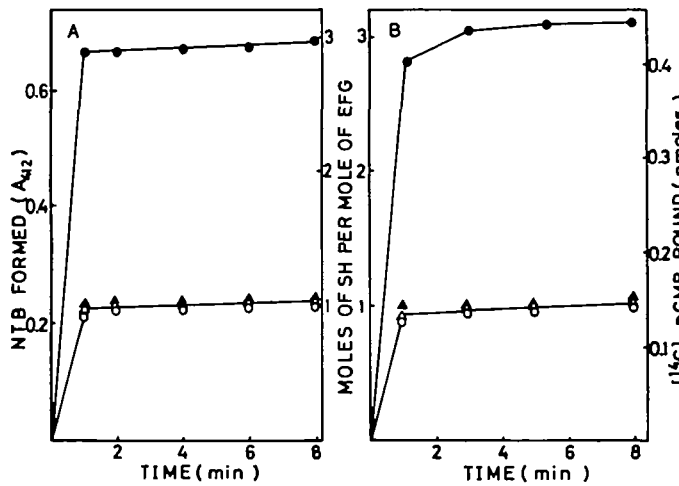

Fig. 1. Titration of sulfhydryl groups of EF-G. In A, 0.72 mg of EF-G was titrated at room temperature with 0.4 mM DTNB in 0.5 ml of 0.1 M Tris-HCl (pH 8.0) in the presence (●) or absence (○) of 6 M urea and 1% Na-DodSO$_4$, and in the latter case with addition of 0.6 mM GDP (▲) or 0.6 mM GTP (△). The reaction was started by the addition of DTNB. The amount of sulfhydryl titrated was calculated from the absorbance at 412 nm of NTB formed using a molar absorbancy of 13.6×10^3 M$^{-1}\cdot$cm^{-1}. In B, 60 μg of EF-G was titrated at 0°C with 18 μM [^{14}C]pCMB (specific activity 12 μCi/μmol) in 0.5 ml of 0.1 M Tris-HCl (pH 8.0) (○). Further additions were 6 M urea (●), 0.6 mM GDP (▲), or 0.6 mM GTP (△). The reaction was started by the addition of [^{14}C]pCMB. At indicated times, 0.1 ml of the sample was withdrawn and filtered on the nitrocellulose membrane filter. The filter was washed 3 times with 10 mM Tris-HCl (pH 7.9) and 10 mM magnesium acetate and the radioactivity was determined.

50% of the activities were lost by treatment of EF-G with 5 μM NEM for 5 min at 0°C.

The effect of NEM on the ability of EF-G to form a binary EF-G·guanine nucleotide complex was also examined. For this experiment, EF-G which had been pretreated with NEM and had lost about 80% of its uncoupled GTPase activity was assayed for the binary complex formation using the gel filtration method according to Hummel and Dreyer (*27*). As shown in Fig. 3, the peak of [^{3}H]radioactivity which coincided with the protein peak appeared at the void volume and was followed by a constant level of [^{3}H]GDP and then by fractions which were deficient in [^{3}H]GDP. The amount of [^{3}H]GDP bound to EF-G was calculated from the area of the peak and the trough as approximately 0.9 mol of EF-G, indicating that the ability to form the binary complex was hardly affected by NEM treatment. A similar result was also obtained by equilibrium dialysis using the pCMB-treated EF-G. EF-G (15 mg/ml) was treated with 0.83 mM pCMB at 0°C for 30 min and then unreacted pCMB was removed by dialysis. In a separate experiment using [^{14}C]pCMB, it was shown that more than 90% of EF-G was labeled with pCMB. As shown in Fig. 4, the pCMB-treated EF-G preserved the ability to form the binary EF-G·GDP complex. A Scatchard plot of the result showed that the pCMB-treated EF-G had 1.2 binding sites for GDP per mole of protein and the dissociation constant was about 4×10^{-4} M. The latter value is to be compared to the 5×10^{-5} M of the untreated EF-G·GDP complex. These results indicated that NEM or pCMB treated EF-G could interact with GDP, but with a somewhat decreased affinity.

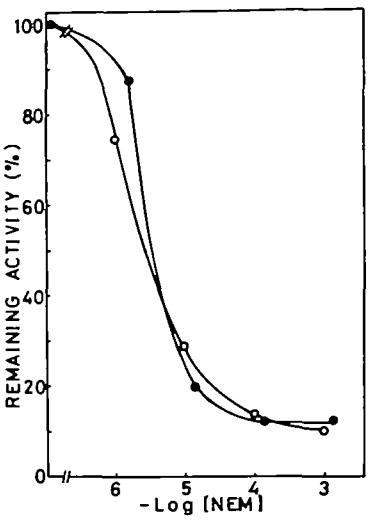

Fig. 2. Effect of NEM on the uncoupled GTPase activity and on the formation of the ternary complex. In the case of GTPase activity (○), EF-G (0.11 mg/ml) was incubated with NEM in 50 μl of 18 mM Tris-HCl buffer (pH 7.6) at 0°C for 5 min and the reaction was stopped by the addition of an excess amount of 2-mercaptoethanol. A 2-μl portion was taken for assay of GTPase activity. In the case of the ternary complex formation (●), 0.23 mg/ml of EF-G was treated with NEM in the above manner and 10-μl aliquots were taken for the assay of the ternary complex formation.

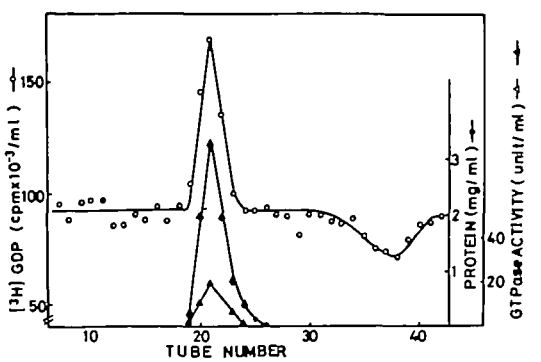

Fig. 3. Formation of a binary complex between GDP and NEM-modified EF-G. A sample of the modified EF-G was chromatographed on a Sephadex G-25 column equilibrated with a buffer containing [³H]GDP. The elution was followed by radioactivity of [³H]GDP (○), protein concentration (●), and remaining activity of EF-G (▲). A similar experiment was carried out with the same amount of unmodified EF-G and the activity (△) was plotted. Details are described under "EXPERIMENTAL PROCEDURES."

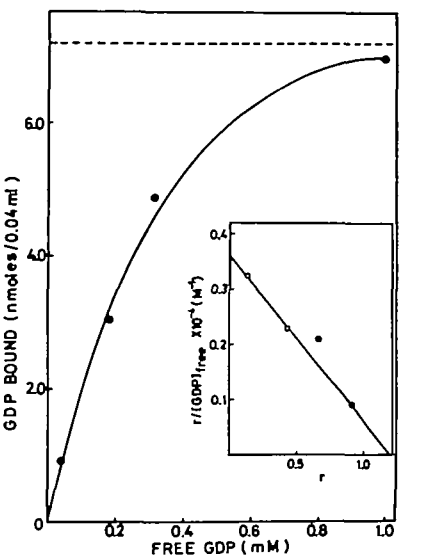

Fig. 4. Binding of GDP to the pCMB-treated EF-G as revealed by equilibrium dialysis. Dotted line indicates the amount of EF-G added. Details are described under "EXPERIMENTAL PROCEDURES." In the inset, the results are shown as a Scatchard plot.

TABLE I. Protection of EF-G from NEM Inactivation. EF-G was incubated with NEM in the presence of guanine nucleotide and ribosomes either alone or in combination, and then assayed for the remaining uncoupled GTPase activity. The details are given under "EXPERIMENTAL PROCEDURES."

Additions	Activity remaining (%)
None	28
GTP	30
Ribosomes	21
GDP and ribosomes	29
GMP-P (CH_2)P and ribosomes	85

Protection of EF-G against NEM Inactivation—EF-G was treated with NEM in the presence or absence of ribosomes and guanine nucleotides, and remaining EF-G activity was determined by the uncoupled GTPase activity. As shown in Table I, the addition of either GTP or ribosomes alone did not protect EF-G from NEM inactivation. In the separate experiments, neither GMP-P(CH_2)P nor GDP alone protected EF-G against NEM inactivation (data not shown). Although the addition of ribosomes did not have any significant

effect, a marked protection was observed by the addtion of both GMP-P(CH_2)P and ribosomes. These results indicate that the reactive sulfhydryl group is not required for binding of guanine nucleotide but probably is essential for interaction with ribosomes.

Difference in Reactivity of the Sulfhydryl Group of EF-G·GTP and EF-G·GDP—Since the affinity of EF-G toward ribosomes was altered by binding with GDP and GTP (*5*, *6*), it was expected that the reactivity of the sulfhydryl group to certain sulfhydryl reagent(s) might differ between EF-G·GDP and EF-G·GTP. To test this possibility, the rate of reaction of the sulfhydryl in EF-G with a hydrophobic sulfhydryl reagent, ANM (*28*) was measured.

As shown in Fig. 5, EF-G reacts rapidly with ANM to yield a fluorescent product having an emission maximum at 440 nm upon exicitation at 350 nm. It was found that the rate of the reaction was affected by the addition of nucleotide ligands. When EF-G was incubated with ANM at 20°C, the modification proceeded very rapidly and was almost complete within 2 to 3 min. On the other hand, the rate was decreased by the addition of 0.1 mM GTP or 0.1 mM GDP. The initial velocity of the reaction of EF-G·GTP and EF-G·GDP with ANM was reduced to about 75% and 50%, respectively, of that of free EF-G. However, the final fluorescence intensity was almost identical in all cases. The effect of 1 mM GMP-P(NH)P was almost the same as that of 0.1 mM GTP.

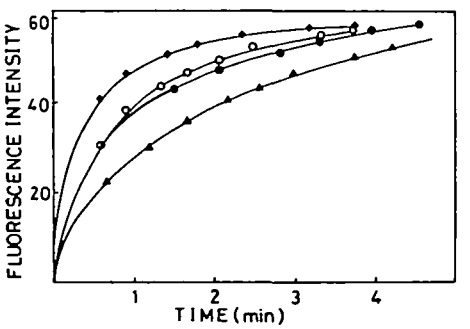

Fig. 5. Kinetics of the reaction of ANM and EF-G. The reaction was carried out as described under "EXPERIMENTAL PROCEDURES" and measured fluorometrically. Excitation at 350 nm and emission at 440 nm. EF-G alone (◆), and EF-G with 0.1 mM GTP (○), 1 mM GMP-P(NH)P (●), or 0.1 mM GDP (▲).

These results suggested that binding of nucleotides induced conformational transition of EF-G near the sulfhydryl group essential for interaction with ribosomes, and the reactivity toward ANM was altered.

DISCUSSION

The main conclusions that emerged from the present investigation are that (1) *E. coli* EF-G contains one reactive sulfhydryl group, (2) this group is not required for the nucleotide binding but is essential for interaction with ribosomes, and (3) the reactivity of the sulfhydryl group toward ANM is modulated by guanine nucleotides. These findings are in line with our previous report that ESR spectra of EF-G, which is spin-labeled specifically at this protein sulfhydryl, changed upon conversion of its ligand from GDP to GTP (*7*).

As is clear from the titration experiments shown in Fig. 1, the addition of guanine nucleotides to EF-G did not protect the reactive sulfhydryl group from DTNB or pCMB. Furthermore, the formation of the binary EF-G·guanine nucleotides complex could be observed with NEM-, and pCMB-modified EF-G (Figs. 3 and 4). With the modified EF-G, the maximal binding of guanine nucleotide was nearly stoichiometric, but the dissociation constant of the modified EF-G·GDP complex seemed to be greater than that of the unmodified EF-G·GDP complex by one order of magnitude. On the other hand, the ability to interact with ribosomes as measured by formation of the ternary EF-G·GMP-P(CH_2)P·ribosome complex or by uncoupled GTPase reaction was rapidly destroyed by modification of the sulfhydryl group (Fig. 2). As expected, the sulfhydryl group was protected against NEM inactivation by the addition of both GMP-P(CH_2)P and ribosomes (Table I). Finally, the reactivity of the sulfhydryl group toward ANM was shown to be different between EF-G·GDP and EF-G·GTP (Fig. 5), suggesting that the reactivity of EF-G·GDP and EF-G·GTP (Fig. 5), suggesting that the reactivity of EF-G toward ribosomes is also modulated by the guanine nucleotide ligands.

Recently, Marsh *et al.* (*29*) reported that the rate of modification of EF-G by DTNB was reduced by the addition of guanine nucleotides, and concluded that the sulfhydryl group of EF-G is

essential for nucleotide binding. However, in view of the present finding, their results can be interpreted as due to the nucleotide-induced change of reactivity of the sulfhydryl group, rather than the direct shielding of the sulfhydryl group by guanine nucleotides.

Rohrbach and Bodley (*17*) also found that the ability of EF-G to form a ternary EF-G·GMP-P(CH_2)P·ribosome complex was irreversibly lost by modification of one cysteine residue with NEM. However, they concluded that the sulfhydryl group is rather essential for the binding of guanine nucleotide since the modification had no effect on the ability of EF-G to form an EF-G·ribosome complex (*17*). The apparent discrepancy between their conclusion and ours is based on the fact that their EF-G·ribosome complex is not the correct intermediate for the formation of the ternary EF-G·GTP·ribosome complex. As we have shown, the formation of the ternary complex occurs through the binary EF-G·GTP complex as an intermediate (*6–8*), and this was supported by more recent observations by Rohrbach and Bodley (*14*) and Baca *et al.* (*13*). Presumably, the nonspecific and nucleotide-independent interaction of EF-G with ribosomes, which may not require the true active site of EF-G, led them to the erroneous conclusion. A similar interaction of EF-2 with ribosomes in the absence of GTP (*30*) was shown to be nonspecific by Mizumoto *et al.* (*31*). In the case of pig liver EF-2, it was also found that the sulfhydryl group is not essential for nucleotide binding but for interaction with ribosomes (Mizumoto, K., a personal communication).

It must be noted that the reactivity of the sulfhydryl group toward ANM was different between EF-G·GDP and EF-G·GTP. This is probably due to the conformational difference between EF-G·GDP and EF-G·GTP as has been previously observed using hydrophobic (*6*) and spin-label (*7*) probes. In the case of the spin-label experiment, the conformational change was also located near the sulfhydryl group essential for the interaction with ribosomes. Since EF-G·GTP and not EF-G·GDP can interact with ribosomes to form a ternary complex, it is reasonable that the conformational change is induced in EF-G around the active site for interaction with ribosomes, *i.e.* near the sulfhydryl group, to increase the reactivity toward ribosomes in the presence of GTP, and, conversely, to reduce it in the presence of GDP.

Therefore, as we have pointed out before (*6–8*), the similarity in the mechanism of EF-Tu- and EF-G-promoted reactions is rather evident. In both cases, the reactivity toward ribosomes is modulated by the species of bound guanine nucleotides through the conformational change which is occurring at or near the reactive protein sulfhydryl.

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