Selective Chemical Modification of Escherichia coli Elongation Factor G

N-Ethylmaleimide Modification of a Cysteine Essential for Nucleotide Binding*

(Received for publication, July 28, 1975)

Michael S. Rohrbach and James W. Bodey

From the Department of Biochemistry, University of Minnesota, Minneapolis, Minnesota 55455

Escherichia coli Elongation Factor G is inhibited irreversibly by the chemical modification of 1 cysteine residue with N-ethylmaleimide. At pH 5.2, this cysteine is approximately 130 times more reactive than β-mercaptoethanol toward N-ethylmaleimide. Inhibition is not prevented by either the ribosome or GTP alone at concentrations approximately equal to that of Elongation Factor G, but in combination they reduce the inhibition by 50%. Increasing the stability of the Elongation Factor G-ribosome-GDP complex by the addition of fusidic acid, completely protects against N-ethylmaleimide inhibition. The modified protein cannot form either the Elongation Factor G-ribosome-GMP-P(CH)2P or the Elongation Factor G-ribosome-GDP-fusidic acid complex. However, the modification had no effect on its ability to form the Elongation Factor G-ribosome complex. These results suggest that the cysteine residue modified by N-ethylmaleimide is at or near the nucleotide binding site.

Elongation Factor G interacts with the ribosome and GTP to catalyze the translocation step of the protein synthesis elongation cycle (2). While the mechanism of action of this protein has been investigated in a number of laboratories, the chemical features of the protein which are essential for its function remain undefined.

Since shortly after its discovery, EF-G (1) has been assumed to contain 1 or more essential cysteine residues. This conclusion was based on the observations that its participation in protein synthesis was abolished by various sulphydryl reagents and depended on the presence of a protecting thiol (2,3). More recently we have reported that EF-G in the absence of a protecting thiol undergoes both reversible and irreversible oxidative inactivation (4). Beyond these preliminary observations, however, there have been no reports which define the reactivity or functional role of this presumably essential cysteine residue (or residues).

In this report we describe the inactivation of EF-G through the selective chemical modification of 1 cysteine residue with low molar excesses of N-ethylmaleimide (MalNEt). Through substrate protection experiments, we also demonstrate that this very reactive cysteine is located at or near the nucleotide binding site on the factor.

* This investigation was supported by Grants GM-17101 and GM-21339 from the National Institutes of Health. This paper is No. XIX in the series: "Studies on Translocation." The preceding paper is Ref. 1.

The abbreviations used are: EF-G, Elongation Factor G; MalNEt, N-ethylmaleimide; GMP-P(CH)2P, (β,γ-mercaptoethanol)guanosine 5'-triphosphate.

EXPERIMENTAL PROCEDURES

Materials—Mid-log cells of Escherichia coli B were purchased from Grain Processing Corp. and were the source of both ribosomes (5) and EF-G which was purified to homogeneity as previously described (6). [3H]GTP (9.35 Ci/mmol) and [3H]MalNEt (230 Ci/mmol) were purchased from New England Nuclear Corp. [3H]GDP (5 Ci/mmol) and [2,3-3H]MalNEt (2.1 mCi/mmol) were obtained from Amersham/Searle Corp. [3H]GMP-P(CH)2P was prepared as described earlier (1). MalNEt was purchased from Sigma Chemical Co. Fusidic acid was a generous gift from Dr. W. O. Godtfredsen of Leo Pharmaceutical Products. All other chemicals were of the highest purity commercially available.

Methods—All MalNEt inhibition reactions (100 µl) contained 295 µM of EF-G and were performed at 35°C unless otherwise stated. Aliquots (2 µl) were withdrawn at timed intervals prior to MalNEt addition and were assayed by quantitation of the EF-G-ribosome-[3H]GTP-fusidic acid complex by Millipore filtration (7) in order to establish the value for 100% activity. Following reaction with MalNEt, aliquots (2 µl) were again withdrawn at time intervals and assayed to determine the extent of inhibition. In all cases, controls incubated for the same time in the absence of MalNEt remained 100% active.

pH and MalNEt Concentration Dependence of Inhibition—The pH dependence of inhibition was determined by reaction of EF-G with 5.09 × 10−6 M MalNEt. Reactions were performed in 50 mM Tris-maleate buffer (pH 5.05 to 8.00) containing 4.5 mM β-mercaptoethanol.

The MalNEt concentration dependence of inhibition was examined at pH 5.2 in 100 mM sodium acetate buffer employing MalNEt concentrations from 1.79 × 10−4 to 1.14 × 10−3 M. Inhibition was performed with 2.29 × 10−4 M [3H]MalNEt and β-mercaptoethanol concentrations from 2 to 47 mM in 100 mM sodium acetate buffer (pH 5.2). Activity was assayed as above with the substitution of [γ-32P]GTP for [3H]GTP in the assay.
Aliquots (25 μl) of the inhibition mixture were then added to 10 μl of bovine serum albumin (6 mg/ml). Twenty-five microliters of cold 20% trichloroacetic acid were added to precipitate the protein. The filters were washed twice with cold 10% trichloroacetic acid, twice with anhydrous ether, and were dried and counted for quantitation of incorporation of [3H]MalNEt.

Identification of Residue Modified—EF-G (11.36 nmol) was reacted in a final volume of 1.1 ml with 4.76 x 10⁻⁴ M [2-3H]MalNEt in 100 mM sodium acetate, pH 5.2, containing 4.5 mM β-mercaptoethanol. Excess reagent was removed by dialysis against 100 mM sodium acetate, pH 5.2. The labeled protein precipitated under these conditions and was collected by centrifugation. The pellet protein was dissolved in 500 μl of 6 N HCl, and was hydrolyzed in vacuo at 100° for 72 hours. The hydrolysate was chromatographed on a Beckman model 119 amino acid analyzer with the effluent diverted to a fraction collector following passage through the colorimeter. Aliquots (600 μl) of the fractions were monitored for radioactivity (8).

A standard sample of β-(ethyl[2,3-3H]succininido)cysteine prepared by the method of Fruton et al. (9) was hydrolyzed and chromatographed in the same manner as the radiolabeled protein.

Protection Against MalNEt Inhibition—All inhibitions were performed as described above using 1.54 x 10⁻⁴ M MalNEt. Each reaction contained 3.25 x 10⁻⁴ M EF-G and when indicated, 4.35 x 10⁻⁴ M [3H]GTP, 4 x 10⁻⁴ M ribosomes, or 4.35 x 10⁻⁴ M [3H]GTP and 4 x 10⁻⁴ M ribosomes. These reactions were repeated with the inclusion of 5 mM fusidic acid in each reaction.

Multiple Function Assays—Samples of EF-G were inhibited with 3.30 x 10⁻⁴ M MalNEt and were assayed as above for their ability to participate in complex formation. The complexes measured were the EF-G-ribosome-GDP-fusidic acid complex formed from either GTP or GDP, and the EF-G-ribosome-GMP-P(CH)₃P complex. The ability to form the EF-G-ribosome complex in the absence of nucleotide and fusidic acid was quantitated by the gel chromatography assay of Lin and Bodley (7) using MalNEt-inhibited [3H]EF-G. The values for per cent inhibition were calculated by dividing the picomoles of complex formed with the MalNEt-modified EF-G by those formed with the unmodified EF-G.

RESULTS

Inhibition of EF-G by N-Ethylmaleimide—The reaction of MalNEt with EF-G under conditions designed to maximize selective labeling, i.e. low molar excesses of MalNEt, exhibited a number of unusual features which were due to intrinsic characteristics of the protein. As we have reported previously, exogenous thiol is required to maintain EF-G activity (4). Since we were unsuccessful in obtaining active protein in the absence of protecting thiol, all reaction solutions contained β-mercaptoethanol at concentrations which greatly exceeded those of MalNEt. Because of this, the MalNEt was consumed rapidly and only the final extent, but not the rate of inhibition, could be measured. In addition, the extent of inhibition was a function of both the MalNEt and β-mercaptoethanol concentrations.

The inhibition of EF-G by MalNEt was markedly pH-dependent. As the pH was lowered to 6.5, the inhibition, measured at fixed concentrations of β-mercaptoethanol and MalNEt, increased. Below pH 6.5 the reaction was pH-independent.

These features of the inhibition are illustrated in Fig. 1 which shows the inhibition of EF-G by increasing concentrations of MalNEt at two pH values. Comparison of the MalNEt concentrations required to obtain maximal inhibition at pH 5.2 (open circles) and 7.4 (closed circles) graphically demonstrates the increased reactivity of the protein at acidic pH values. In addition, the MalNEt concentration dependence can be seen at both pH values.

One other important characteristic of this inhibition is also shown in this figure, namely, the presence of a MalNEt-insensitive fraction of EF-G. This MalNEt-insensitive EF-G is not a function of the pH as the same maximal level of inhibition was obtained at both pH 5.2 and 7.4.

Our initial attempts to increase the extent of inhibition by altering the reaction temperature, changing the ionic conditions, or making multiple additions of MalNEt proved unsuccessful. However, preincubation of EF-G at 37° in buffer containing 35 mM as opposed to 5 mM β-mercaptoethanol activated the MalNEt-insensitive EF-G, and 90% inhibition could be achieved subsequently (data not shown). In order to obtain these levels of inhibition in the presence of 35 mM β-mercaptoethanol, correspondingly higher concentrations of MalNEt were required. These concentrations were too high to be practical when radiolabeled MalNEt was used and we have therefore not “activated” the EF-G in the experiments which follow.

Since the inhibition reaction was so rapid, the incorporation of [3H]MalNEt could not be correlated with inhibition by removing aliquots for analysis as a function of time. However, by performing the inhibition at increasing levels of β-mercaptoethanol, the final extent of inhibition and incorporation is reduced progressively. In this manner incorporation can be correlated with inhibition (Fig. 2). As shown in the figure by extrapolation to 100% inhibition, incorporation of 0.97 mol of MalNEt would result in complete inactivation.

In addition to providing a means for the correlation of inhibition and incorporation, the relative reactivity of the protein compared to β-mercaptoethanol can be calculated from the increased reactivity of the protein at acidic pH values.

![Fig. 1. MalNEt concentration dependence of inhibition at two pH values. Inhibition by increasing concentrations of MalNEt was measured as described under “Experimental Procedures.” The pH values of the inhibition reaction were: 5.2 (O) and 7.4 (●).](attachment://fig1.png)

![Fig. 2. Incorporation of [3H]MalNEt into EF-G as a function of the per cent inhibition. The incorporation was performed at pH 5.2 and was quantitated by trichloroacetic acid precipitation. The β-mercaptoethanol concentrations employed to give the levels of inhibition shown are described under “Experimental Procedures.”](attachment://fig2.png)
this data. Division of the \( \beta \)-mercaptoethanol/EF-G ratio at which 50% maximal inhibition occurs by the molar excess of MalNEt yields the relative reactivity. In this way, at pH 5.2, EF-G was calculated to be 130 times more reactive toward MalNEt than was \( \beta \)-mercaptoethanol.

Identification of Modified Residue—In order to identify the amino acid residue in EF-G modified by MalNEt a sample of \([2,3-^{14}C] \) MalNEt-inactivated protein was hydrolyzed in vacuo at 110° for 72 hours with 6 n HCl. The hydrolysate was chromatographed on a Beckman model 119 amino acid analyzer. The effluent was collected and fractions were monitored for radioactivity. A standard sample of \( S\)-(ethy[2,3-^{14}C]succinimidio)cysteine prepared by the method of Fruton et al. (9) was hydrolyzed and chromatographed in the same manner. Seventy-two hours of hydrolysis were employed to ensure complete conversion of the \( S\)-(N-ethylsuccinimidio)cysteine to \( S\) (succinyl)cysteine (10).

The radioactivity derived from the \( ^{14}C \)-labeled MalNEt-inactivated protein eluted in a single peak at 42 ml. No radioactivity was observed in any other position of the chromatography. The \( ^{14}C \)-labeled \( S\)-(succinyl)cysteine also eluted at 42 ml, indicating that cysteine is the amino acid modified in EF-G.

Protection against MalNEt Inhibition—The ability of the ribosome, GTP, and fusidic acid, either individually or in combination, to protect EF-G against MalNEt inhibition was examined as described under "Methods." As shown in Table I, significant protection against inhibition was observed under these conditions only when both the ribosome and GTP were present. In the absence of fusidic acid the EF-G-ribosome-GDP complex is relatively labile and only 50% protection was observed. Fusidic acid is known to increase the stability of this complex (11), and addition of the antibiotic resulted in essentially complete protection against inhibition. These results indicate that the modification occurred at or near one of the binding sites of one of these molecules. The site containing the modified cysteine was identified as described below.

Site of MalNEt Modification—The activity measurement employed in this investigation was based on the formation and quantification of the EF-G-ribosome-[\( ^{3}H \)]GDP-fusidic acid complex. This complex can be formed by EF-G and the ribosome in the presence of fusidic acid by a single round of hydrolysis of [\( ^{3}H \)]GTP (12). Modification of EF-G which would prevent either the binding of ribosomes, nucleotide, or fusidic acid, or the hydrolysis of GTP to GDP would result in the observed inhibition. In order to determine which of these sites contained the modified cysteine, the ability of the MalNEt-inhibited EF-G to participate in the complexes listed in Table II was examined.

### Table I

**Protection against MalNEt inhibition**

The reaction conditions are described under "Experimental Procedures."

| Reaction additions | Per cent protection | + Fusidic acid | + Fusidic acid |
|-------------------|---------------------|---------------|---------------|
| None              | 0                    | 5             | 97            |
| GTP               | 0                    | 5             | 12            |
| Ribosome          | 0                    | 3             | 12            |
| GTP + ribosome    | 0                    | 3             | 12            |

The substitution of [\( ^{3}H \)]GDP for [\( ^{3}H \)]GTP in the assay obviates the requirement for hydrolysis prior to complex formation. As shown in the table, the same degree of inhibition was observed when either [\( ^{3}H \)]GTP or [\( ^{3}H \)]GDP was employed. This would suggest but not prove that inhibition was not due to the modification of a catalytically essential residue.

The ability of the modified enzyme to form the EF-G-ribosome-[\( ^{3}H \)]GMP-P(CH)P and [\( ^{3}H \)]EF-G-ribosome complexes can be used to define which of the three binding sites contains the modified residue. The formation of the [\( ^{3}H \)]EF-G-ribosome complex requires neither nucleotide nor fusidic acid, while the EF-G-ribosome-[\( ^{3}H \)]GMP-P(CH)P complex does not require fusidic acid. Only the formation of the [\( ^{3}H \)]EF-G-ribosome complex is unaffected by MalNEt modification suggesting that inhibition is due to the inability of the modified enzyme to participate in the binding of nucleotide.

### DISCUSSION

The modification of EF-G with MalNEt was complicated by the required presence of \( \beta \)-mercaptoethanol in at least a 1500-fold excess over EF-G. In spite of this large excess of \( \beta \)-mercaptoethanol, inhibition of EF-G was observed at low molar excesses of \( N \)-ethylmaleimide, indicating that the protein cysteine was more reactive toward \( N \)-ethylmaleimide than was \( \beta \)-mercaptoethanol.

Although rapid inhibition was observed under these reaction conditions, approximately 40% of the EF-G appeared to be refractory to MalNEt inhibition. The subsequent observation that preincubation of the protein in high concentrations of \( \beta \)-mercaptoethanol converted the MalNEt-insensitive fraction to an MalNEt-sensitive form parallels the results of a previous study of the effects of air oxidation on activity in which we reported that air oxidation led to inactivation which could be reversed by the addition of \( \beta \)-mercaptoethanol (4). In fact, reactivation was observed during activity measurements due to the high \( \beta \)-mercaptoethanol/EF-G ratio in the assay. It is likely that the MalNEt-insensitive EF-G is protein which had oxidized upon storage, thereby making the active cysteine unavailable for modification. During the assay for activity, this oxidized EF-G is reduced giving rise to the observation that a fraction of the EF-G appears to be both fully active and MalNEt-insensitive. This hypothesis was substantiated further by the observation that the per cent of MalNEt-insensitive EF-G is a function of the length of storage. Preparations of EF-G which were stored for a shorter time then that used in

### Table II

**Ability of MalNEt-modified EF-G to form complexes**

The ability of MalNEt-modified EF-G to participate in complex formation was quantitated as described under "Experimental Procedures." The inhibition observed in the formation of the EF-G-ribosome-GDP-fusidic acid complex from GTP was set arbitrarily at 100% maximal inhibition. This value was used to normalize the observed inhibition of the other complexes.

| Complex measured | Per cent maximal inhibition |
|------------------|----------------------------|
| EF-G-ribosome-GDP-fusidic acid from GTP | 100 |
| EF-G-ribosome-GDP-fusidic acid from GDP | 108 |
| EF-G-ribosome-GMP-P(CH)P | 103 |
| EF-G-ribosome | 0 |
Selective Chemical Modification of Elongation Factor

this study contained smaller percentages of MalNEt-insensitive protein.\textsuperscript{3}

The presence of the unreactive fraction of EF-G had no effect on either the outcome or the interpretation of experiments reported here other than limiting the maximal level of inhibition of 60 to 70%. However, the observation that EF G tends to undergo a reversible oxidation upon storage may have significant effects on the selective modification of other residues at the active site. It is possible that due to this oxidation, normally accessible residues near the cysteine may become inaccessible to reagent. This limitation will have to be born in mind in subsequent modifications of the active site (or sites) of EF-G.

The relative reactivity of the cysteine residue modified by MalNEt exhibited an unusual pH dependence; its relative reactivity increased as the pH was lowered from neutrality of pH 6.5. Since the reaction with MalNEt occurs via nucleophilic substitution, the unprotonated form of cysteine is the more reactive species. This pH dependence suggests that the reactive cysteine has a lowered pHₐ.

In order to draw meaningful conclusions about the nature of the active center of a protein from chemical modification studies, two criteria have to be fulfilled: (a) the modification must be selective, \textit{e.g.} a linear relationship must exist between inhibition and incorporation of the modifying reagent; (b) modification must occur at or near the active center. The modification of EF-G with MalNEt described here fulfills both of these requirements. Incorporation of radiolabeled MalNEt parallels the observed inhibition. Extrapolation to 100% inactivation indicates that 0.97 mol of MalNEt would be incorporated per mol of EF-G.

Protection against inhibition by equal molar concentrations of GTP and ribosomes indicate that the reactive cysteine is located at or near the active center of the protein. In its broadest terms, this active center contains sites for the interaction with the ribosome, nucleotide, and fusidic acid. The identification of the site containing the modified cysteine was elucidated from the ability of the modified protein to form the EF-G:ribosome:GDP-fusidic acid, EF-G:ribosome:GMP-7CH₃P, and EF-G:ribosome complexes. The observation that the MalNEt modified EF G could not form the first two complexes but was fully competent in the formation of the third indicated that the modified cysteine was located at the nucleotide binding site.

These results represent the first functional assignment of an amino acid residue in EF-G. It is interesting to note that EF-Tu also contains a reactive cysteine at its nucleotide binding site (14). It may be that this is a general characteristic of the nucleotide binding sites of the protein synthesis factors.

REFERENCES

1. Skar, D. C., Rohrbach, M. S., and Bodley, J. W. (1975) Biochemistry 14, 3922-3926
2. Nishizuka, Y., and Lipmann, F. (1966) Arch. Biochem. Biophys. 116, 344-351
3. Kaziro, Y., Inoue, N., Kuriki, Y., Mizumoto, K., Tanaka, M., and Kawakita, M. (1969) Cold Spring Harbor Symp. Quant. Biol. 34, 385-393
4. Rohrbach, M. S., Bodley, J. W., and Mann, G. G. (1975) J. Biol. Chem. 250, 6831-6836
5. Bodley, J. W. (1969) Biochemistry 8, 465-475
6. Rohrbach, M. S., Dempsey, M. E., and Bodley, J. W. (1974) J. Biol. Chem. 249, 5094-5101
7. Highland, J. H., Lin, L., and Bodley, J. W. (1971) Biochemistry 10, 4404-4409
8. Bodley, J. W., Zieve, F. J., Lin, L., and Zieve, S. T. (1970) J. Biol. Chem. 245, 5656-5661
9. Fruton, J. S., Smyth, D. G., and Nagamatsu, A. (1963) J. Am. Chem. Soc. 82, 4600-4607
10. Smyth, D. G., Blumenfeld, O. O., and Konigsberg, W. (1964) Biochem. J. 91, 580-588
11. Bodley, J. W., Zieve, F. J., Lin, L., and Zieve, S. (1969) Biochem. Biophys. Res. Commun. 37, 437-443.
12. Bodley, J. W., Zieve, F. J., and Lin, L. (1970) J. Biol. Chem. 245, 5662-5667
13. Miller, D. L., Hachmann, J., and Weissbach, H. (1971) Arch. Biochem. Biophys. 144, 115-121

\textsuperscript{3}M. S. Rohrbach and J. W. Bodley, unpublished observation.
Selective chemical modification of Escherichia coli elongation factor G. N-Ethylmaleimide modification of a cysteine essential for nucleotide binding.
M S Rohrbach and J W Bodley

J. Biol. Chem. 1976, 251:930-933.

Access the most updated version of this article at http://www.jbc.org/content/251/4/930

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/251/4/930.full.html#ref-list-1