CHEMICAL EVIDENCE FOR THE INVOLVEMENT OF HISTIDYL RESIDUES IN THE FUNCTIONING OF *ESCHERICHIA COLI* ELONGATION FACTOR Tu

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1. Introduction

Peptide chain elongation factor Tu (EF-Tu) catalyses GTP-dependent binding of aminoacyl-tRNA to the A site of ribosomes through the intermediate formation of a ternary EF-Tu · GTP · aminoacyl-tRNA complex (reviewed [1]).

We have tried [2,3] to identify the regions in both EF-Tu and the aminoacyl-tRNA involved in their mutual binding and proposed a model of the interaction between EF-Tu · GTP and aminoacyl-tRNA. The model implies that the 3′-terminus of the aminoacyl-tRNA interacts with EF-Tu, and that the SH-group of cysteine or/and some amino acid residue(s) located close to this cysteine in the aminoacyl-tRNA binding site of the factor could be involved in the interaction with the 3′-terminus of aminoacyl-tRNA [3]. The observation that the tryptic peptide of *E. coli* EF-Tu containing the cysteine residue important for aminoacyl-tRNA binding is rich in histidine residues and particularly the fact that two of these residues are located symmetrically around the cysteine [4] led us to investigate the role of histidine residues in the function of EF-Tu.

Here we describe studies on the inactivation of EF-Tu · GDP by diethylpyrocarbonate, a relatively specific histidine reagent [5], and by photooxidation in the presence of the rose bengal dye which is the most selective method available for the modification of histidine residues in proteins [6]. The results of these experiments and the amino acid analysis of the photooxidized factor suggest that histidine residues are involved in the binding of EF-Tu to aminoacyl-tRNA and/or ribosomes.

2. Materials and methods

The preparation of crystalline EF-Tu · GDP, and tRNA from *E. coli* B and of [^{3}H]Phe-tRNA (9240 cpm/pmol) was described in [2]. L-[^{3}H]Phenylalanine (21 Ci/mmol) and [8-^{3}H]guanosine 5′-diphosphate, ammonium salt (15.4 Ci/mmol, 7650 cpm/pmol) were from the Radiochemical Centre, Amersham. Phosphoenolpyruvate, pyruvate kinase and poly(U) were from Calbiochem. Guanosine 5′-diphosphate, trisodium salt was purchased from Merck; ATP, disodium salt was from Reanal, Hungary. Diethylpyrocarbonate (DEPC) was obtained from Bayer AG, and rose bengal B was from Lachema, Czechoslovakia.

Ribosomes from *E. coli* B cells washed 3 times with 0.5 M NH_4Cl were prepared according to [7], except that the cells were sonicated in 0.01 M Tris–HCl (pH 7.5), 0.01 M magnesium acetate, 0.02 M NH_4Cl and 5 mM 2-mercaptoethanol (2-ME).

The fraction with EF-G activity obtained by DEAE–Sephadex column chromatography of *E. coli* B S-100 supernatant ([8], method B) was precipitated by ammonium sulphate between 38–59% saturation. The precipitate was dissolved in 0.02 M Tris–HCl (pH 7.5), 5 mM 2-ME and 0.25 M sucrose, and stored in dry ice. Just before use an aliquot was diluted with 1 vol. H_2O, heated for 4 min at 55°C, the precipitate removed by centrifugation and the opalescent supernatant diluted with 1 vol. H_2O. This preparation of EF-G was completely free of EF-Tu activity.

2.1. *Assay of Ef-Tu activities*

The exchange of [^{3}H]GDP with EF-Tu · GDP in the absence of EF-Ts was measured using the nitrocellulose membrane filter technique [8] in buffer I

(0.04 M Tris–HCl (pH 7.6), 0.01 M magnesium acetate, 0.16 M NH_4Cl and 5 mM 2-ME) for 20 min at 4°C.

The EF-Tu activity of aminoacyl-tRNA transfer to ribosomes was assayed in buffer I by the Millipore technique [9].

Phenylalanine polymerization activity of EF-Tu was assayed in buffer I under similar conditions as in [10].

2.2. *Reaction of EF-Tu with DEPC*

Crystalline EF-Tu · GDP (7 nmol) was washed with 42% saturated ammonium sulphate [3] and dissolved in 600 μl buffer II (10 mM Tris–HCl (pH 7.0), 5 mM $MgCl_2$, 100 mM KCl and 10 μM GDP). The pH was adjusted to 6.0 with 65 μl 1 M sodium cacodylate, 0.05 M $MgCl_2$ buffer, and the solution was treated with 10 mM DEPC. (DEPC was diluted with methanol before use; final methanol concentration in the reaction mixture: 5%.) After 60 min at 4°C, the solution was brought to 60% saturation with solid ammonium sulphate, the precipitate washed with 0.8 ml buffer II containing 60% ammonium sulphate and dissolved in 180 μl buffer II containing 20 μM GDP. Control sample of EF-Tu was treated with 5% methanol only.

Hydroxylamine treatment of the inactivated or control EF-Tu. The reaction mixture (120 μl) contained 14 μM either DEPC-treated or control EF-Tu (see above) and 1 M hydroxylamine (adjusted to pH 7.0 with KOH). After incubation at 25°C for 30 min, 300 μl buffer II were added and EF-Tu was separated from the reagent at 4°C by the ammonium sulphate precipitation and washing procedure above. The sediment was dissolved in 0.2 ml 50 mM Tris–HCl (pH 7.6), 10 mM magnesium acetate, 10 μM GDP and 1 mM 2-ME, and the sample was assayed for EF-Tu activity. Controls contained no hydroxylamine.

2.3. *Photooxidation of EF-Tu*

Crystalline EF-Tu · GDP was washed as above and photooxidation of EF-Tu · GDP was done using rose bengal as a photosensitizer [11]. The reaction mixture (75 μl) containing 32 μM EF-Tu · GDP and 8 μM rose bengal in 20 mM Tris–HCl (pH 7.6), 10 mM magnesium acetate, 5 mM 2-ME and 10 μM GDP (buffer III) was cooled on ice, aerated by stirring and irradiated by a 500 W slide projector at 32 cm. Aliquots were withdrawn at appropriate intervals, diluted 6.5 times with buffer III (without GDP) in the dark and analysed for EF-Tu activity. Dark controls were treated identically but left in the dark.

3. Results

3.1. *Inactivation of EF-Tu by DEPC*

The action of DEPC at acidic pH is relatively specific for histidyl residues [5,12] and additional evidence for histidine involvement can be provided by data on reactivation of DEPC-treated enzyme with hydroxylamine [13]. Fig.1 shows the results from such an experiment with EF-Tu. The reaction of native EF-Tu · GDP with DEPC at pH 6.0 results in complete loss of its polymerization activity (fig.1, □). The GDP binding and/or exchange activity of DEPC-inactivated EF-Tu was only partly reduced (~37% inhibition, table 1) suggesting that other functions(s) of EF-Tu

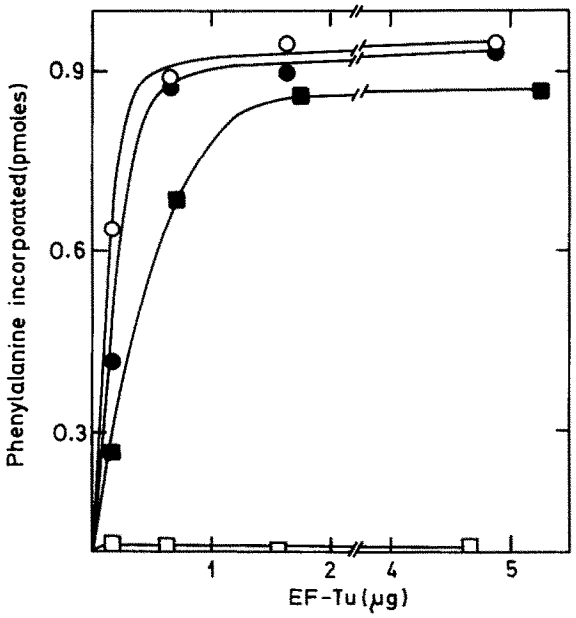

Fig.1. Poly(U)-dependent phenylalanine polymerization activity of ethoxyformylated EF-Tu · GDP and of ethoxyformylated EF-Tu · GDP reactivated with hydroxylamine. The reaction mixtures (0.1 ml) containing 90 μg ribosomes, 16 μg poly(U), 0.5 mM GTP, 0.4 mM ATP, 1.7 mM phosphoenolpyruvic acid, 6 μg pyruvate kinase, 6 μg EF-G preparation, 1 μg [^{3}H]Phe-tRNA (1.1 pmol phenylalanine) and varying levels of either control EF-Tu · GDP (○), control hydroxylamine-treated EF-Tu · GDP (●), ethoxyformylated EF-Tu · GDP (□) or ethoxyformylated hydroxylamine-treated EF-Tu · GDP (■) in buffer I were incubated for 20 min at 35°C and the hot trichloracetic acid-precipitable radioactivity was measured in an Isocap liquid scintillation spectrometer (Nuclear Chicago). The value of 0.03 pmol obtained in the absence of EF-Tu has been subtracted.

Table 1
[^{3}H]GDP ⇌ EF-Tu · GDP exchange activity of ethoxyformylated EF-Tu · GDP and of ethoxyformylated EF-Tu · GDP reactivated with hydroxylamine

EF-Tu · GDP treated with		[^{3}H]GDP bound to EF-Tu (pmol)
DEPC	Hydroxylamine	
–	–	8.44
–	+	6.07
+	–	5.35
+	+	5.39

5 μl (1.6 μg) EF-Tu · GDP treated as indicated and 93 pmol [^{3}H]GDP in 0.1 ml total vol. buffer I were incubated for 20 min at 4°C and the protein-bound [^{3}H]GDP was determined as in section 2. The values in the table are not corrected for the dilution by cold GDP contained in EF-Tu solutions (see section 2)

than binary complex formation is responsible for the inability of the factor to promote phenylalanine polymerization. The results in fig.2 confirm this assumption. The ability of EF-Tu to catalyse binding of aminoacyl-tRNA to ribosomes is likewise destroyed by DEPC treatment (fig.2, □) indicating that the binding site for aminoacyl-tRNA and/or ribosomes is much more strongly affected by the reagent that is the binding site for GDP/GTP.

Treatment of ethoxyformylated EF-Tu · GDP with 1 M hydroxylamine for 30 min results in a high but incomplete restoration of two of the original EF-Tu functions, polymerization and Phe-tRNA binding, which are recovered to the same extent (fig.1,2, ■). The high concentration of DEPC (10 mM) used in the experiment fully destroyed both these functions (fig.1,2, □). Incompleteness of reactivation of DEPC-treated EF-Tu · GDP by hydroxylamine may thus suggest that either some dicarbethoxylation of essential histidyl residue(s) in EF-Tu occurred (dicarbethoxyhistidyl residues formed by reaction with excess DEPC are resistant to the action of hydroxylamine [14]) or that some irreversible inactivation was caused also by modification of other groups, such as lysyl residues. Further, as mentioned in table 1, GDP binding activity of EF-Tu is also affected by DEPC; treatment of the inactivated factor with hydroxylamine only slightly restored this activity (table 1). This might explain the incompleteness of regeneration of the two other EF-Tu functions which both depend on the efficiency of protein–guanosine nucleotide complex formation [1]. Nevertheless, the large extent of reactivation produced by hydroxylamine demonstrated in fig.1,2 support the idea that inhibition by diethylpyrocarbonate of EF-Tu function in elongation is mainly due to the modification of histidyl residues at the binding site for aminoacyl-tRNA and/or ribosomes.

3.2. *Photooxidation of EF-Tu*

Upon irradiation with visible light, EF-Tu · GDP in the presence of 8 μM rose bengal is rapidly inactivated (fig.3, ●,■), whereas irradiation of the protein in the absence of the dye (□,○) or incubation of EF-Tu · GDP with rose bengal without irradiation (dark controls) did not show any substantial effect on the factor. The

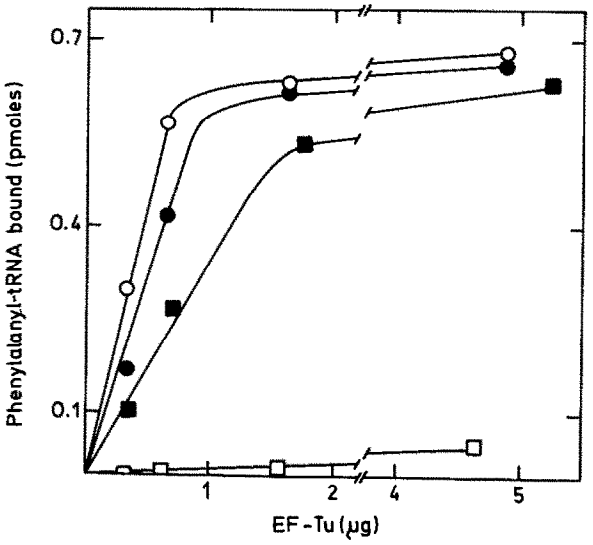

Fig.2. Capacity of ethoxyformylated EF-Tu · GDP and of ethoxyformylated EF-Tu · GDP reactivated with hydroxylamine to promote Phe-tRNA binding to ribosomes. To the reaction mixture (20 μl) containing 0.5 mM GTP, 10 mM phosphoenolpyruvic acid and 3 μg pyruvate kinase in buffer I preincubated for 5 min at 30°C and chilled to 4°C were added 1 μg [^{3}H]Phe-tRNA (1.1 pmol phenylalanine) and varying levels of either control EF-Tu · GDP (○), control hydroxylamine-treated EF-Tu · GDP (●), ethoxyformylated EF-Tu · GDP (□) or ethoxyformylated hydroxylamine-treated EF-Tu · GDP (■) and the samples (final vol. 35 μl) were incubated for additional 5 min at 30°C. After chilling to 4°C, 50 μl mixture containing 8 μg poly(U), 60 μg ribosomes and 70 μg total tRNA in buffer I (preincubated for 45 min at 30°C) were added, the whole mixture (85 μl) incubated for 30 min at 4°C and the radioactivity bound to ribosomes determined (see section 2 and fig.1). The value of 0.025 pmol obtained in the absence of EF-Tu has been subtracted.

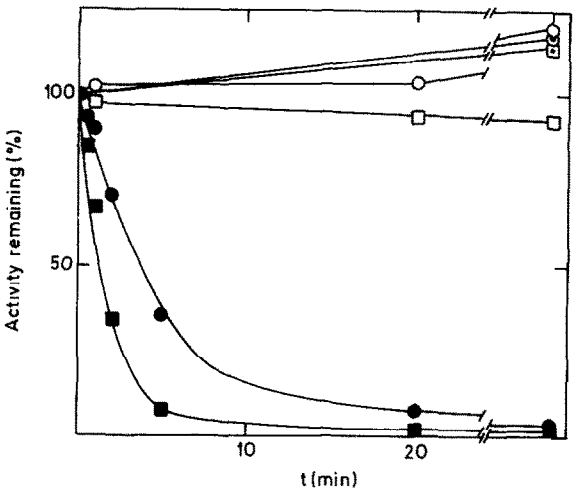

Fig.3. Time course of photoinactivation of EF-Tu. EF-Tu · GDP was photooxidized (●,■) or irradiated in the absence of the dye (○,□) or incubated with rose bengal without irradiation (dark controls, ⊙,⊡), for the times indicated and the capacity to bind [^{3}H]GDP (●,○,⊙) or to promote [^{3}H]-Phe-tRNA binding to ribosomes (■,□,⊡) were measured as described in table 1 and fig.2. Reaction mixtures for GDP binding contained 0.63 μg EF-Tu · GDP; 100% activity corresponded to 3.4 pmol [^{3}H]GDP bound, whereas in Phe-tRNA binding experiments 2.5 μg EF-Tu · GDP was used and 100% activity corresponded to 0.72 pmol [^{3}H]Phe-tRNA bound.

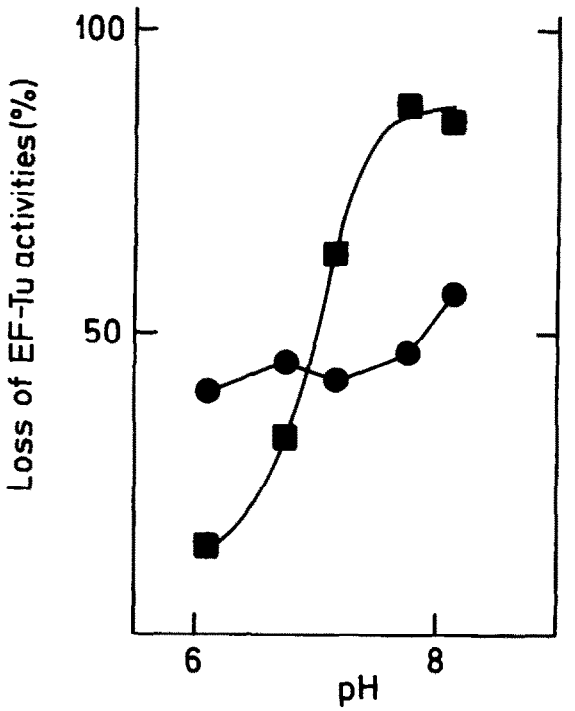

Fig.4. pH dependence of rose bengal-sensitized photoinactivation of EF-Tu · GDP. EF-Tu · GDP was prepared as described in section 2 and the pH adjusted as indicated by addition of 0.1 vol. 1 M sodium cacodylate, 0.05 M $MgCl_2$. Samples were irradiated for 2.5 min and EF-Tu activities were determined (see section 2 and fig.3). (●) [^{3}H]GDP binding activity; (■) [^{3}H]Phe-tRNA binding to ribosomes.

time course of the photoinactivation of EF-Tu funtions shows that the capacity to promote binding of aminoacyl-tRNA to ribosomes is lost much faster than its capacity to bind and/or exchange GDP (fig.3).

Fig.4 shows the effect of the pH on the rate of photoinactivation of EF-Tu · GDP sensitized by rose bengal. The curve of the loss of the activity of the factor to catalyse binding of phenylalanyl-tRNA to ribosomes, as a function of the pH, is sigmoidal, with an inflection near pH 7.0. The photooxidation of histidine alone shows an almost identical sigmoidal pH profile, the protonated form of histidine being resistant to oxidation [11] and the result in fig.4 thus suggests that one or more histidyl residue(s) are being photooxidized which are important for the binding site of the factor for aminoacyl-tRNA and/or ribosomes.

The curve of the rate of loss of GDP binding activity of EF-Tu as a function of pH has a different shape. This suggests that loss of GDP binding activity may be due to photooxidation of a different amino acid residue, or that if a histidyl residue(s) is (are) also essential for this function, it may be protected by bound GDP.

4. Discussion

The properties of the aminoacyl-tRNA and/or ribosome binding site of *E. coli* EF-Tu were investigated by chemical modification of EF-Tu · GDP. Treatment of EF-Tu with DEPC or photooxidation of the factor in the presence of rose bengal dye selectively impairs the activity of EF-Tu to bind to aminoacyl-tRNA and/or ribosomes, whereas the GDP binding and/or exchange activity of EF-Tu is much less affected. The separate inhibition of aminoacyl-tRNA and GDP binding activities of the factor by DEPC as well as by photooxidation argues for the localization of the effects and suggests that they are direct and not caused by general structural changes of the protein. The finding that the inactivation of ethoxyformylated EF-Tu is largely reversed by treatment with hydroxylamine and the results of the pH dependence of EF-Tu · GDP inactivation by photooxidation together with the demonstration that EF-Tu in the presence of GTP and aminoacyl-tRNA was protected from photo-

oxidation (not shown) indicate that one or more histidyl residues are essential for the binding of the factor to aminoacyl-tRNA and/or ribosomes.

The involvement of histidyl residues in the binding of the factor with GDP is not clear, but cannot be excluded because such a histidine might be protected from modification by DEPC or from destruction by photooxidation by bound GDP. However, the hydroxylamine reactivation experiment as well as the pH dependence of the loss of the GDP binding activity of EF-Tu in the photooxidation experiment show that the integrity of other amino acid residues, which can be modified by DEPC or destroyed by photooxidation (e.g., methionine, tyrosine, cysteine or tryptophane), might also be important for this function.

The only amino acid residue which has so far been identified as essential (reviewed [1]) or at least important [2,15] for complex formation of EF-Tu with aminoacyl-tRNA is the cysteine located at position 81 from the N-terminus of the protein [16]. Although DEPC is relatively specific for histidyl residues at pH 6, we also studied the effect of the reagent on EF-Tu · GDP pretreated with *p*-chloromercuribenzoate which reversibly blocks free SH groups of the factor (unpublished) to exclude the possibility that a cysteine might be the primary site of DEPC action or be affected with histidyl residues. However, treatment of EF-Tu · GDP with *p*-chloromercuribenzoate did not protect the factor from DEPC inactivation, indicating that other groups than the cysteine residues must be responsible for the loss of the activity of EF-Tu to bind to aminoacyl-tRNA and/or ribosomes due to DEPC action. Similar results were obtained in photooxidation experiments (unpublished). Additional evidence for the role of histidyl residue(s) in the activity of EF-Tu comes from the amino acid analyses of the photooxidized EF-Tu · GDP. Only histidyl residues (~55%) were lost by irradiation of EF-Tu · GDP in the presence of rose bengal for 10 min.

Results obtained by proton nuclear magnetic resonance and photooxidation which appeared [17] suggest that histidine residues are involved in the binding of *Thermus thermophilus* EF-Tu with aminoacyl-tRNA and/or ribosomes as well as with guanosine nucleotides.

It has been shown recently that histidyl residues are important also for other enzymatic systems forming complexes with aminoacyl-tRNA during peptide chain elongation, e.g., for ribosomal peptidyl-transferase center [18,19] and for aminoacyl-tRNA synthetase [20]. Histidine residue(s) might thus be generally important for the binding of any protein which in the course of protein biosynthesis forms a complex with an aminoacyl-tRNA. Whether the regular CCA-amino acid 3′-terminal sequence of aminoacyl-tRNA, which seems to be particularly involved in the formation of complexes with binding proteins [3], represents the counterpart for histidyl residue(s) in the proteins remains to be elucidated.

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