A SULPHYDRYL GROUP IS NOT ESSENTIAL FOR THE BINDING OF GDP TO ELONGATION FACTOR Tu

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1. Introduction

Studies on the reactions of the sulphydryl groups of polypeptide elongation factor Tu (EF-Tu) from *Escherichia coli* [1,2] have led to certain generally accepted conclusions. These are that one sulphydryl group (SH 1) is involved in the binding of GDP or GTP, and a second (SH 2) in the interaction with aminoacyl-tRNA [3,4]. However, recent results on the structure of EF-Tu · GDP from *E. coli* have indicated that the nucleotide binding region is located in one protein domain and the cysteine residues in the other [5]. We report below some studies with the nucleotide free elongation factor Tu ($EF\text{-}Tu_f$) from *Bacillus stearothermophilus* which would support this observation and indicate that a sulphydryl group is not essential for the binding of GDP.

2. Materials and methods

Nucleotide free EF-Tu from *B. stearothermophilus* was prepared as in [6]. Removal of low molecular weight mercaptans was achieved by passing 1 ml samples of protein solution through a column (1.5 × 22 cm) of Sephadex G-25 (Pharmacia) equilibrated in nitrogen-flushed 50 mM Tris · HCl (pH 7.4) (buffer A). *N*-Ethylmaleimide (MalNEt) was obtained from Serva and was used as an aqueous solution. [^{14}C]MalNEt (Amersham) had spec. act. 170 MBq/mmol and was brought into solution in buffer A by the procedure suggested by the supplier. By means of competition experiments with freshly prepared solutions of unlabelled MalNEt, it was found that only ~35% of the [^{14}C]MalNEt could react with SH-groups probably due to partial polymerisation of the radioactive material.

All modification reactions were performed in buffer A. Reaction with 5,5′-dithiobis (2-nitrobenzoic acid) ($(Nbs)_2$) was carried out by adding 25 µl 10 mM reagent in buffer A to a 1 ml sample of 10 µM protein solution in a cuvette. A_{412} was recorded with time.

For GDP binding activity measurements, 1.5 ml 13 µM $EF\text{-}Tu_f$ was reacted with 50 µl 10 mM $(Nbs)_2$; at suitable time intervals 10 µl samples were removed into 200 µl buffer A containing 10 µM [^{3}H]GDP (40.7 GBq/mmol) and 10 mM $MgCl_2$, incubated for 1 min at room temperature and the protein-bound [^{3}H]GDP determined by nitrocellulose filter assay [6].

The procedure for the modification of sulphydryl groups with [^{14}C]MalNEt was as in [6].

Equilibrium dialysis was performed in plexiglass chambers through a membrane filter (Schleicher and Schuell, RC 51, Batch 14/892) at 12°C. The buffer system was 20 mM Epps-Na^+, 1 mM DTE, 10 mM $MgCl_2$, KCl to I = 0.2 (pH 8.13). The procedure will be detailed elsewhere (in preparation).

3. Results

Elongation factor Tu from *B. stearothermophilus* contains 2 SH-groups [6] which by analogy with the *E. coli* factor are designated SH 1 and SH 2. Figure 1A shows that both SH-groups can be modified with

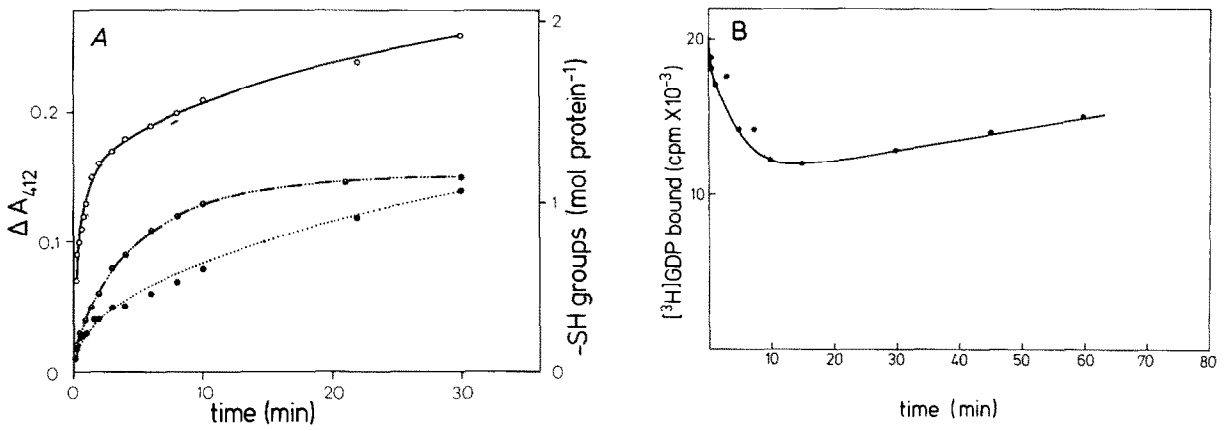

Fig.1. Reaction of EF-Tu$_f$ with $(Nbs)_2$. (A) 1 ml 10 μM EF-Tu$_f$ in buffer A was reacted with 50 μl 10 mM $(Nbs)_2$ as in section 2 and the A_{412} monitored. No addition (○——○); plus 0.2 mM GDP (—· · ·○—· · ·); plus 2 mM $MgCl_2$ and 0.2 mM GDP (● · · · ●). (B) GDP binding activity of 13 μM EF-Tu$_f$ during reaction with 3.2 mM $(Nbs)_2$ solution measured as in section 2.

$(Nbs)_2$ within 30 min when the protein is free of metal ions and GDP [7]. The presence of either GDP or MgGDP has a marked effect on the reactivity of 1 SH-group (SH 1) so that after 30 min only the SH 2 group has been modified. This is in agreement with [2] where 1 SH-group in EF-Tu · GDP from *E. coli* was found to react with $(Nbs)_2$ whereas in the presence of excess EDTA (to generate EF-Tu$_f$ in situ) 2 SH-groups react. Figure 1B shows the change in GDP binding activity of EF-Tu$_f$ during the course of $(Nbs)_2$ modification. In the first 10 min of the reaction, when on average 1.5 SH-groups/protein molecule are modified, the activity apparently drops to 63% of the starting value but after 30 min, when 2 SH-groups have been modified, the activity has reverted to its original value. Figure 1B shows that even with 2 bulky 5-thio-2-nitrobenzoate groups bound to the protein 60%, if not 100%, of the GDP binding activity of EF-Tu is still present. Preliminary experiments show that the initial decline of activity is the result of an altered reaction rate of association so that, unlike with unmodified EF-Tu$_f$, the reaction is not complete within 1 min.

GDP binding activity of *E. coli* EF-Tu has been reported lost [1,2] only when high concentrations of MalNEt (10 mM) are used for the modification of SH-groups whereas the [^{14}C]tRNA binding ability is lost rapidly on incubation in a 1 mM solution of the reagent. We followed the eventual change in GDP binding activity of EF-Tu from *B. stearothermophilus* during reaction with 1 mM MalNEt at room temperature. Under these conditions EF-Tu · GDP is completely stable and EF-Tu$_f$ loses ~10–15% of its activity during 60 min of reaction (fig.2). When the incorporation of [^{14}C]MalNEt into protein was monitored under the same conditions, it was found

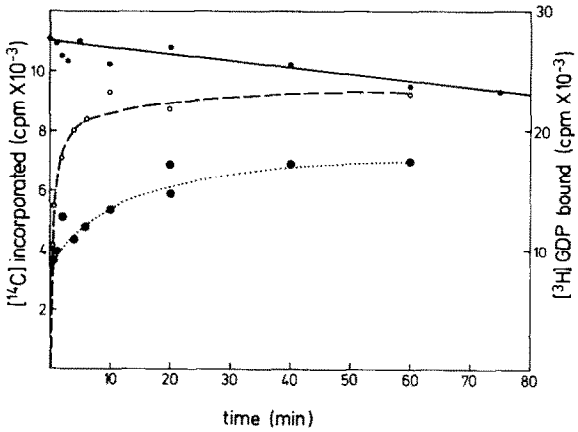

Fig.2. Reaction of EF-Tu$_f$ with *N*-ethylmaleimide. 200 μl 17.2 μM EF-Tu$_f$ was reacted with 40 μl <0.5 mM [^{14}C]-MalNEt in buffer A (– –○–) and in the presence of 30 μM GDP (· · ·●· · ·) and the incorporation of ^{14}C was measured. In a parallel experiment a sample of the same EF-Tu$_f$ solution was reacted with 0.5 mM unlabelled MalNEt and the [^{3}H]GDP binding activity of the protein was measured (—●——●—).

that two molecules of the reagent were bound to the protein. Here also, as with $(Nbs)_2$ modification, the presence of GDP reduces the reaction rate considerably. The competition experiments with MalNEt (section 2) demonstrate that the unlabelled reagent also binds to the protein.

Since the GDP binding activity of MalNEt-treated EF-Tu as measured by the nitrocellulose filter method is somewhat reduced, we determined the binding constant of GDP to the modified protein by equilibrium dialysis. Figure 3 shows the formation curve for the binding of GDP to MalNEt-treated EF-Tu. At 70% saturation the curve levels off, which means that the stoicheiometry was not changed as the starting material was only 65% active. From the midpoint of the curve ($\bar{\nu} = 0.35$) a binding constant of 8.3×10^6 can be calculated. Under the same conditions a binding constant of 1.6×10^7 for unmodified EF-Tu from *B. stearothermophilus* is found.

4. Discussion

The modifications by $(Nbs)_2$ and MalNEt of the sulphydryl groups of $EF\text{-}Tu_f$ proceed rapidly and are inhibited by GDP. This finding is in agreement with results obtained with EF-Tu · GDP from *E. coli* [1,2] and *B. stearothermophilus* [6]. This would appear to support the belief that a free sulphydryl group (SH 1) is required for the binding of GDP or GTP [1–4]. However, SH-modification in the absence of GDP produces a protein which differs only slightly from native protein in its ability to bind GDP to nitrocellulose filters. Equilibrium dialysis shows that modification does not affect the stoicheiometry of the GDP binding and reduces the binding constant only by a factor of ~2.

From these experiments we conclude that the inhibition of SH1 modification by GDP is due neither to the direct participation of the sulphydryl group in the binding of the nucleotide nor to the presence of cysteine in the binding site. Two possible models could explain the results:

1. Binding of GDP sterically blocks access to SH 1;
2. GDP induces conformational changes in part or all of the protein which makes reaction of SH 1 less favourable.

Results obtained in this laboratory (Frank and A.W., to be published) on the amino acid sequence of EF-Tu from *B. stearothermophilus* indicate that its 2 sulphydryl groups occupy positions in the primary structure analogous to SH 1 and SH 2 in the *E. coli* factor sequence [8,9]. Thus a direct comparison of the functions of the sulphydryl groups of the two proteins is permissible. This leads us to favour the second model since the binding of GDP to EF-Tu causes a general 'tightening' of the protein structure [10,11], and the GDP binding site and the cysteine residues appear to be located in different structural domains [5]. Support for our contention that SH 1 is not directly involved in GDP binding, is contained in [3] that EF-Tu from *Thermus thermophilus* contains no free sulphydryl groups and has a very high affinity for GDP.

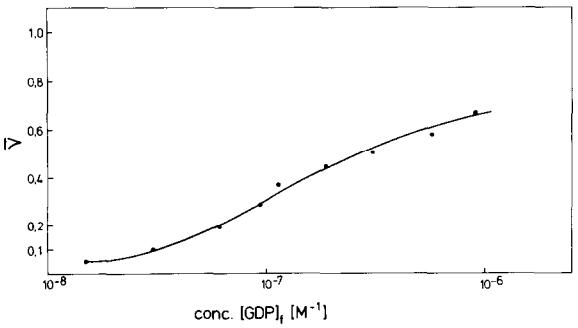

Fig.3. Formation curve for complex between GDP and MalNEt-treated EF-Tu from *B. stearothermophilus*. 39 μM $EF\text{-}Tu_f$ was reacted with 0.5 mM MalNEt for 15 min at room temperature, then a 10-fold excess of DTE was added. The formation curve was determined by equilibrium dialysis of 0.94 μM modified protein using 0.1–3 μM [^{3}H]GDP.

$\bar{\nu} = [EF\text{-}Tu \cdot GDP]/[EF\text{-}Tu \cdot GDP] + [EF\text{-}Tu_f]$

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