A ^{1}H NMR STUDY OF THE *ESCHERICHIA COLI* ELONGATION-FACTOR Tu WITH GUANINE NUCLEOTIDES AND THE ANTIBIOTIC KIRROMYCIN

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1. Introduction

The functional state of the elongation-factor Tu from *Escherichia coli* during the elongation cycle is controlled by guanine nucleotides (review [1]). The differences in affinity of EF-Tu · GTP and EF-Tu · GDP towards aa-tRNAs, ribosomes and EF-Ts is considered to be due to conformational differences which depend on the guanine nucleotide that is bound to EF-Tu. A variety of experiments have been carried out that demonstrate conformational changes of EF-Tu upon substitution of GDP by GTP: hydrogen/tritium and hydrogen/deuterium exchange experiments [2,3], fluorescence spectroscopy employing reporter groups [4,5] as well as intrinsic tryptophane fluorescence [6], ESR [7,8] and proteolytic digestion [15]. However, the optical rotatory dispersion spectra of EF-Tu · GDP and EF-Tu · GTP are identical [3,6]. This finding indicates that the conformational differences seen by other techniques must be rather subtle, since they do not lead to a change in the relative amount of secondary structure elements. The antibiotic kirromycin is known to inhibit protein biosynthesis by complex formation with EF-Tu and interfering with its various conformational states [9–13]. This effect could also be demonstrated by ESR and trypsin-induced cleavage experiments [14–16].

The object of this study was to investigate the conformations of EF-Tu · GDP and EF-Tu · GTP in absence and presence of kirromycin by ^{1}H NMR. ^{1}H NMR spectra have so far only been obtained for EF-Tu from *Thermus thermophilus* [17,18] or EF-Tu from *E. coli* complexed with tRNA [20], since EF-Tu from *E. coli* alone is unstable at room temperature. We have circumvented the stability problem by doing all experiments at 1°C. The low field spectrum of EF-Tu shows several resolved exchangeable resonances in addition to the broad envelope of the CONH and aromatic resonances. Conversion of EF-Tu · GDP to EF-Tu · GTP leads to characteristic changes which in part can be simulated by addition of kirromycin to EF-Tu · GDP indicating, that indeed kirromycin makes EF-Tu · GDP more EF-Tu · GTP-like, as was suggested on the basis of kinetic investigations on the EF-Tu–guanine nucleotide interaction [13] and of proteolytic digestion experiments [16].

Abbreviations: NMR, nuclear magnetic resonance; ESR, electron spin resonance; EF-Tu, elongation-factor Tu; SDS–PAGE, sodium dodecylsulphate–polyacrylamide gel electrophoresis DSS, 3-(trimethylsilyl)-propionic acid; HPLC, high pressure liquid chromatography; aa-tRNA, aminoacyl-transfer ribonucleic acid; M_r, relative molecular mass

2. Materials and methods

5′-GDP, 5′-GTP, phosphoenolpyruvate and pyruvate kinase were purchased from Boehringer (Mannheim). Kirromycin was prepared as in [10]. EF-Tu was isolated from *E. coli* MRE 600 (Public Health Laboratory Service) exactly as in [19] with the modification that all buffers used during the purification contained 0.1 μM GDP. After the AcA 44 chromatography (LKB) fractions containing pure EF-Tu as judged by SDS–PAGE were loaded onto a DEAE-cellulose (Whatman DE-52) column (0.5 × 5 cm). The column was rinsed extensively with 0.05 M KCl in buffer A (0.03 M K-phosphate (pH 7.2), 10 mM $MgCl_2$, 0.1 mM 1.4 dithioerytritol, 0.1 μM GDP). EF-Tu was subsequently eluted with 0.3 M KCl in buffer A. The solution thus obtained was ~0.5–1.5 mM in EF-Tu. The concentration of EF-Tu was determined using $\epsilon^{280} = 32900$ ($A^{280\,nm}_{1\%} = 7.6$, M_r 43 225). The nucleotide content

of EF-Tu was measured employing HPLC over a reverse-phase amino column (Riedel de Haen) with 0.7 M $NH_4H_2PO_4$ solution (pH 4.5) as elutant. Only those EF-Tu samples were used for further experiments that contained >0.7 mol GDP/mol EF-Tu · GDP/GTP conversion was carried out in buffer A with a 2-fold excess of phosphoenolpyruvate, 25% the amount of GTP, both with respect to the EF-Tu concentration, and 0.1 mg/ml pyruvate kinase, at 0°C for 30 min. Under these conditions 75–90% of EF-Tu · GDP was converted to EF-Tu · GTP depending on the EF-Tu sample. If kirromycin was present a higher excess of phosphoenolpyruvate was used in order to compensate for kirromycin-induced GTP hydrolysis.

NMR spectra were accumulated at 1°C on a Bruker WH 270 instrument in correlation mode for 15–30 h and referenced downfield from DSS by use of water as secondary standard. For the broad exchangeable resonances at the low field edge our sweep parameters, 1800 Hz in 1 s, practically corresponded to a normal cw-scan. No deviations occurred after these peaks and the spectra could be plotted directly without correlation, reducing artificial oscillations at the sweep start otherwise produced by the correlation procedure from scans running close to the strong water signal. Spectra containing sharp lines, e.g., from free excess nucleotides, were correlated with the Bruker correlation program. Blank spectra of buffer were subtracted from the sample spectra in order to suppress baseline curvature and oscillation produced by the strong water signal.

3. Results and discussion

The ^1H NMR spectrum of EF-Tu · GDP in water shows several resolved peaks on the low field edge of the envelope of the exchangeable and aromatic resonances (fig.1). The resolved peaks easily exchange with D_2O. Protein resonances have been postulated in this region on the basis of spectra of mixtures of EF-Tu and tRNAs [20].

3.1. *GDP/GTP conversion*

Upon conversion of GDP to GTP by addition of pyruvate kinase and phosphoenolpyruvate the lowest field peak is detected at somewhat higher fields and a characteristic difference spectrum appears at 10–14 and ~7.6 ppm (fig.2). It involves ~5–10 protons when calibrated by the H8-resonance of the excess free GDP. After spontaneous hydrolysis of GTP which was measured by HPLC in a parallel experiment under

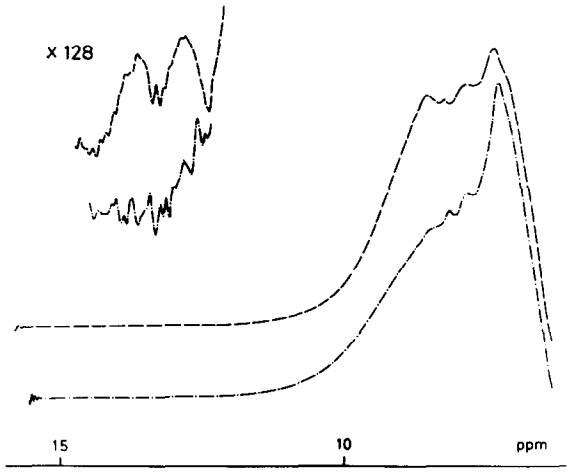

Fig.1. Low field ^1H NMR spectrum of EF-Tu · GDP in H_2O (– –) and D_2O (–·–): 1.3 mM EF-Tu · GDP, 5.8 mM phosphoenolpyruvate, 1.9 mM GDP, buffer A, 0.3 M KCl.

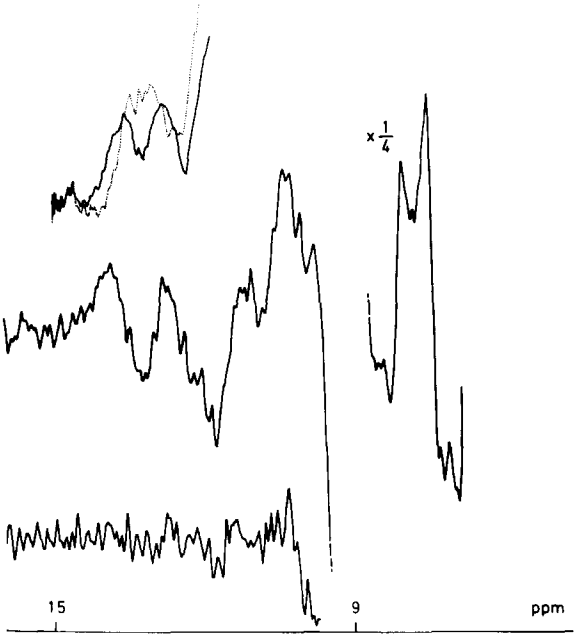

Fig.2. Low field ^1H NMR spectra of EF-Tu · GDP (upper trace ——) and EF-Tu · GTP (upper trace ·····) in H_2O. Difference spectrum EF-Tu · GDP–EF-Tu · GTP (middle trace). Control difference spectrum of the same sample before conversion of GDP to GTP (EF-Tu · $GDP_{initial}$) and after hydrolysis of GTP to GDP (EF-Tu · GDP_{final}) (lower trace) (cf. fig.3).

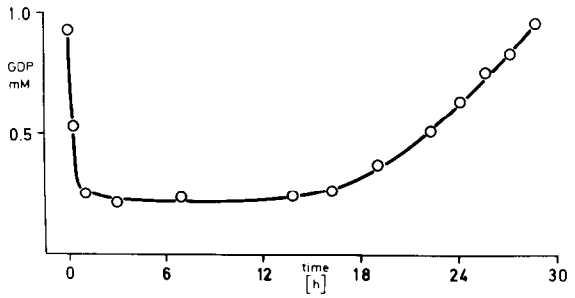

Fig.3. To a 1.3 mM EF-Tu · GDP solution, which had been pre-incubated in buffer A, 0.3 M KCl in the presence of 1.9 mM GDP and 5.8 mM phosphoenolpyruvate for 12 h at 1°C, 0.18 mg pyruvate kinase/ml was added. The solution was then incubated at 1°C. Aliquots (5 μl) were withdrawn and analyzed by HPLC for its EF-Tu · GDP content: at 3–12 h nearly 80% of EF-Tu is present as EF-Tu · GTP; after 30 h EF-Tu is present as EF-Tu · GDP. NMR spectra were recorded in a parallel experiment during the pre-incubation, over 3–12 h and after 30 h.

identical conditions (fig.3), the difference spectrum disappears again indicating that it has not been induced irreversibly by the pyruvate kinase. Control experiments also ruled out the possibility that the difference spectrum was produced by small pH changes upon nucleotide conversion. Preliminary experiments show that the resonances at 13.6 and 12.7 ppm are broadened by addition of 0.11 mM $MnCl_2$ (fig.4). At the same Mn^{2+} levels the H8 and NH_2 peaks of free GDP are also broadened in our solution conditions. Since the only tight Mn^{2+} binding site of EF-Tu · GDP is associated with the nucleotide [8], we conclude that the exchangeable low field protons monitoring the conformation of EF-Tu · GDP and EF-Tu · GTP are in the vicinity of the nucleotide. Further assignment of these resonances is in progress.

3.2. *Effect of kirromycin*

The lowest field peak of EF-Tu · GDP and EF-Tu · GTP in the presence of kirromycin appears at the same position as for EF-Tu · GTP without kirromycin (fig.5) demonstrating that with respect to this

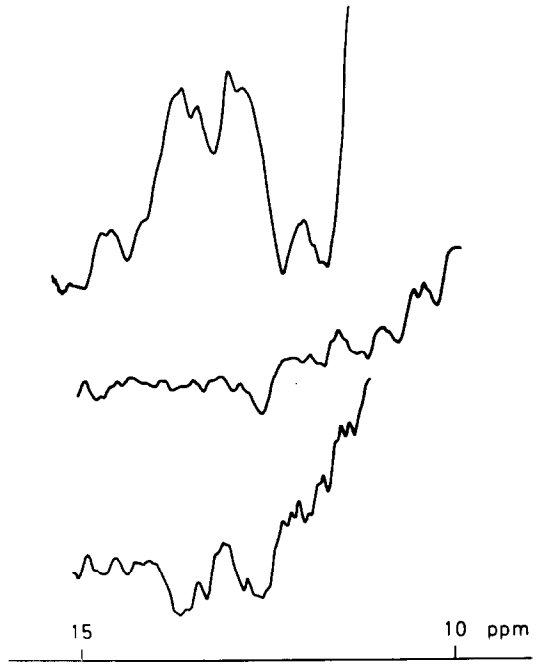

Fig.4. Mn^{2+} dependence of the low field ^1H NMR spectrum of EF-Tu · GDP. Reference spectrum without Mn^{2+} (upper trace). Difference spectra in the presence of 11 μM $MnCl_2$ minus reference spectrum (middle trace) and in the presence of 110 μM $MnCl_2$ minus reference spectrum (lower trace). EF-Tu at, 413 μM in buffer A, 0.3 M KCl.

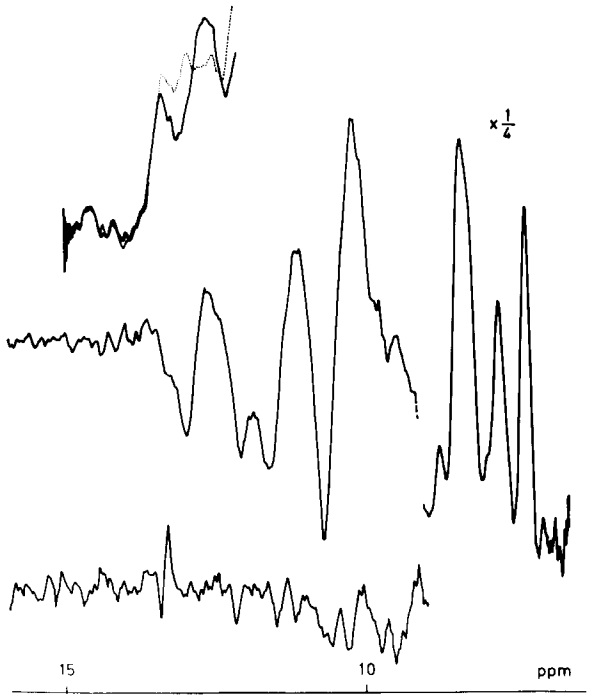

Fig.5. Low field ^1H NMR spectra of EF-Tu · GDP (upper trace ——) and EF-Tu · GTP (upper trace ·····) in the presence of 1.9 mM kirromycin. Difference spectrum EF-Tu · GDP–EF-Tu · GTP (middle trace). Control difference spectrum EF-Tu · $GDP_{initial}$–EF-Tu · GDP_{final} (lower trace) (cf. fig.2). EF-Tu · GDP at 1.1 mM, 14 mM phosphoenolpyruvate, 42 μM GDP, buffer A, 0.3 M KCl.

resonance kirromycin can bring about a similar change as the conversion of GDP to GTP. At 11–13.5 and ~7.6 ppm, however, kirromycin does not effect the difference spectrum EF-Tu · GDP/EF-Tu · GTP, indicating that the antibiotic cannot mimick all of the conformational changes which are due to the GDP/GTP conversion. This is in agreement with ESR and water relaxation studies [14]. It is tempting to speculate, however, that the similar effect of kirromycin and GTP on the lowest field resonance is related to the inhibitory action of the antibiotic. This suggestion is confirmed by preliminary NMR results in the presence of pulvomycin, which does not change the low field resonances of EF-Tu · GDP. Furthermore no changes of the low field spectrum are observed upon conversion of GDP to GTP in the presence of pulvomycin, indicating that the antibiotic freezes the EF-Tu · GDP conformation. The results support the idea that the function of the antibiotic is mediated by conformational changes of EF-Tu: kirromycin stabilizes a conformation of EF-Tu which is similar to the one of EF-Tu · GTP, regardless of which nucleotide is bound to the factor. Pulvomycin, however, stabilizes a conformation which is similar to the one of EF-Tu · GDP, again regardless of which nucleotide is bound.

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