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Nucleotide Binding and GTP Hydrolysis by Elongation Factor Tu from *Thermus thermophilus* As Monitored by Proton NMR†

Stefan Limmer,‡ Christian O. A. Reiser, Norbert K. Schirmer, Norbert W. Grillenbeck, and Mathias Sprinzl*
Laboratorium für Biochemie, Universität Bayreuth, Postfach 10 12 51, W-8580 Bayreuth, FRG
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ABSTRACT: Proton NMR experiments of the GTP/GDP-binding protein EF-Tu from the extremely thermophilic bacterium *Thermus thermophilus* HB8 in H_2O have been performed paying special attention to the resonances in the downfield region (below 10 ppm). Most of these downfield signals are due to hydrogen bonds formed between the protein and the bound nucleotide. However, three downfield resonances appear even in the nucleotide-free EF-Tu. The middle and C-terminal domain (domain II/III) of EF-Tu lacking the GTP/GDP-binding domain gives rise to an NMR spectrum that hints at a well-structured protein. In contrast to native EF-Tu, the domain II/III spectrum contains no resonances in the downfield region. Several downfield resonances can be used as a fingerprint to trace hydrolysis of protein-bound GTP and temperature effects on the EF-Tu·GDP spectra. NMR studies of the binding of guanosine nucleotide analogues (GMPPNP, GMPPCP) to nucleotide-free EF-Tu have been carried out. The downfield resonances of these complexes differ from the spectrum of EF-Tu·GTP. Protected and photolabile caged GTP was bound to EF-Tu, and NMR spectra before and after photolysis were recorded. The progress of the GTP hydrolysis could be monitored using this method. The downfield resonances have been tentatively assigned taking into account the known structural and biochemical aspects of EF-Tu nucleotide-binding site.

The elongation factor Tu (EF-Tu)[1] is a member of the group of GTP/GDP-binding proteins. This group includes translation, initiation, and elongation factors (Kaziro, 1978), signal-transducing G-proteins (Gilman, 1987), proteins involved in protein transport and secretion (Balch, 1990; Rapoport, 1990), and the *ras* gene family (Barbacid, 1987). The hydrolysis of the bound GTP to GDP and the associated conformational change lead to the deactivation of the protein (signal "off"), whereas the binding of GTP leads to an active conformation (signal "on") which enables the protein to interact with the respective effector.

The elongation factor Tu in its GTP conformation promotes the binding of aminoacyl-tRNA (aa-tRNA) to the bacterial ribosome during polypeptide chain elongation. The nucleotide exchange factor, EF-Ts, facilitates the dissociation of GDP from EF-Tu·GDP and subsequent binding of GTP. EF-Tu·GTP can then interact with aa-tRNA, and the thus formed EF-Tu·GTP·aa-tRNA complex binds to the mRNA-programed ribosome. Following GTP hydrolysis, triggered by association with the ribosome, EF-Tu·GDP dissociates from aa-tRNA and ribosome. This functional cycle of EF-Tu resembles that of other GTP/GDP-binding proteins (Bourne et al., 1990) and is important for kinetic control of the fidelity of translation (Thompson & Karim, 1982; Thompson et al., 1986).

The tertiary structure of bacterial EF-Tu was determined by X-ray analysis of the protein crystals originating from *Escherichia coli* (Jurnak, 1985; la Cour et al., 1985). EF-Tu is composed of three structural domains. Domain I, also referred to as the G domain, is the GTP/GDP-binding domain which shares sequence (Bourne et al., 1991) and structural homology with other GTP/GDP-binding proteins, including the *ras* gene product p21 (Jurnak, 1990a,b). Little is known about the structure of the domain II and the C-terminal do-

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* To whom correspondence should be addressed.
‡ On leave of absence from Sektion Physik, Universität Leipzig, FRG.

[1] Abbreviations: DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; EF-Tu, elongation factor Tu; $EF\text{-}Tu_f$, nucleotide-free EF-Tu; GTP and GDP, guanosine 5′-triphosphate and guanosine 5′-diphosphate; GMPPNP, guanosine 5′-(β,γ-imido)triphosphate, GMPPCP, guanosine 5′-(β,γ-methylene)triphosphate; caged GTP, P^3-1-(2-nitrophenyl)ethylguanosine 5′-triphosphate; FID, free induction decay; HMQC, heteronuclear multiple-quantum coherence.

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main III. The structures of these parts of the EF-Tu are poorly resolved in the models derived from X-ray analysis (Jurnak et al., 1989, 1990b; Nyborg & la Cour, 1989; Clark et al., 1990). Recently the domain II/III has been characterized spectroscopically and biochemically using a genetically engineered domain II/III from *E. coli* (Pieper et al., 1990) as well as the domain II/III prepared by limited proteolysis of *Thermus thermophilus* EF-Tu with *Staphylococcus aureus* V8 protease (Peter et al., 1990). The elongation factor Tu from the thermophilic organism has high sequence homology to the *E. coli* counterpart (Seidler et al., 1987; Kushiro et al., 1987).

Protein structure determination by NMR and by X-ray diffraction allows comparison of corresponding structures in single crystals and in a noncrystalline state. This is important since the solution conditions for NMR studies may coincide with the natural, physiological environment of the protein.

The conformational changes during GTP hydrolysis of p21·GTP were determined recently by X-ray analysis (Schlichting et al., 1990) using the Laue method (Hajdu & Johnson, 1990). It is also possible to examine dynamic processes by means of NMR spectroscopy. However, the X-ray analysis up to now provides the only means to obtain the three-dimensional coordinates of virtually all atoms of a large globular protein (larger than 20 kDa) at high resolution.

Redfield and Papastavros (1990) performed NMR studies of the phosphate binding loop of isotopically ^{15}N-labeled p21 and identified amide resonances which are shifted far downfield (i.e., in the region below 10 ppm). These downfield shifts are presumed to be due to strong hydrogen bonds of the NH protons with the β-phosphate oxygens of GDP. The authors raised the question whether these unusually large downfield shifts generally occur in the phosphoryl-binding loop of other GTP/GDP-binding proteins.

Because of the large size of EF-Tu (45 kDa), the resolution of NMR signals in the upfield region is fairly low. Thus we concentrated our attention on the downfield region of the NMR spectrum to see whether resonances occur here that can serve as a fingerprint for nucleotide interaction in EF-Tu, as in the case with p21 (Redfield & Papastavros, 1990). We also wanted to look for spectral alterations during GTP hydrolysis with which assertions could be made about interactions between amino acids of the G domain and various guanine nucleotides. Conclusions about certain specific contributions of the G domain to the NMR spectra can be drawn on the basis of a comparison of the spectra of the intact molecule and the domain II/III. The procedure presented here represents a fairly simple NMR method for characterizing the above-mentioned phenomena in the case of such a high molecular weight protein, which can be applied to other nucleotide-binding proteins.

Materials and Methods

GDP, GTP, GMPPNP, and DNaseI from bovine pancreas (grade II, 2000 units/mg) were obtained from Boehringer (Mannheim, Germany). GMPPCP was from Sigma (Deisenhofen, Germany) and caged GTP was purchased from Calbiochem (Frankfurt, Germany). Lysozyme from hen's egg white (100 kilounits/mg) and all other chemicals used were of the best grade available from Merck (Darmstadt, Germany). Q Sepharose Fast Flow, Sephacryl S200 HR, and CM Sepharose-Cl 6B were obtained from Pharmacia (Freiburg, Germany).

T. thermophilus HB8 cells were grown to the mid-log phase in a modified Castenholz medium (Castenholz, 1969) at 73 °C. Cultures were harvested 3 h after inoculation, yielding 100–120 g (wet weight) from 50 L of culture. The cells were frozen immediately at −70 °C.

EF-Tu from *T. thermophilus* was assayed as described by Arai et al. (1972) and purified as follows: 130 g of cells were washed twice with 500 mL of 50 mM Tris-HCl, pH 7.5 (20 °C), containing 5 mM EDTA, 1 mM β-mercaptoethanol, 20 μM GDP, 100 μM phenylmethanesulfonyl fluoride, and 5% (v/v) glycerol and suspended in 450 mL of the same buffer. Lysozyme (60 mg) was added, and the suspension was stirred for 2 h at 4 °C. The resulting spheroplasts-containing mixture was made 30 mM in $MgCl_2$, treated with 5.5 mg of DNaseI, and stirred for 20 min at 4 °C. Complete cell breakage was performed by nitrogen decompression using a cell-disruption bomb (Parr Instrument Co., Moline, IL). To remove cell debris, the homogenate was centrifuged at 20000*g* for 30 min at 4 °C and subsequently centrifuged at 110000*g* for 3 h. After centrifugation, the clear supernatant was applied to a Q Sepharose Fast Flow anion-exchange column (5 × 50 cm) equilibrated with 50 mM Tris-HCl, pH 7.5 (20 °C), 10 mM $MgCl_2$, 1 mM β-mercaptoethanol, 20 μM GDP, 20 μM phenylmethanesulfonyl fluoride, and 5% (v/v) glycerol and was washed with 2 L of the same buffer. The column was then developed with a 2 × 2 L linear gradient from 0–500 mM KCl in the same buffer. The fractions corresponding to the peak containing the EF-Tu activity were pooled and precipitated with ammonium sulfate to 70% saturation. The ammonium sulfate precipitate was recovered by centrifugation and dissolved in a minimal volume of a buffer containing 50 mM Tris-HCl, pH 7.5 (20 °C), 10 mM $MgCl_2$, 50 mM KCl, 1 mM β-mercaptoethanol, 20 μM GDP, 20 μM phenylmethanesulfonyl fluoride, and 5% (v/v) glycerol. The solution was loaded on to a Sephacryl S200 HR gel-permeation column (5 × 100 cm), preequilibrated, and eluted with the same buffer. The EF-Tu containing fractions were pooled and concentrated by precipitation with ammonium sulfate at 70% saturation. The precipitate was collected by centrifugation, dissolved in a small volume of a buffer containing 100 mM Tris-HCl, pH 7.5 (20 °C), 20 mM $MgCl_2$, 100 mM KCl, 1 mM β-mercaptoethanol, 20 μM GDP, 20 μM phenylmethanesulfonyl fluoride, and 5% (v/v) glycerol and dialyzed against the same buffer. The purified protein was stored in 50% (v/v) glycerol at −20 °C.

Nucleotide-free EF-Tu was prepared as follows: 50 mg of EF-Tu·GDP in 8 mL of buffer containing 50% (v/v) glycerol was diluted 5-fold with 10 mM $(NH_4)H_2PO_4$, pH 5.65, 5 M urea, 10 mM β-mercaptoethanol, and 40 μM phenylmethanesulfonyl fluoride (buffer A). The pH was carefully adjusted to 5.65 with diluted phosphoric acid. This protein solution was applied to a CM Sepharose-CL 6B column (1 × 5 cm) equilibrated with buffer A and washed with the same buffer. Nucleotide-free EF-Tu was obtained by elution with buffer B (buffer A + 200 mM KCl, pH 7.5). After chromatography, the EF-Tu-containing fractions were pooled and, in order to avoid precipitation of protein during dialysis, diluted with buffer B to a protein concentration of <1 mg/mL. Urea was immediately removed by dialysis against 50 mM borate, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, and 0.01% (w/v) NaN_3. The nucleotide-free EF-Tu had a GDP-binding activity of 21 000 units/mg.

Domain II/III of the *T. thermophilus* EF-Tu was prepared as previously described (Peter et al., 1990). For preparation of EF-Tu nucleotide complexes, nucleotide-free EF-Tu was incubated with a 1.2 molar excess of nucleotide. Protein was concentrated by ultrafiltration using Ultrafree-CL 10 000 NMWL units (Millipore, Eschborn, Germany).

Table I: Chemical Shift Values of Downfield ^1H Resonances

sample	peak[a] A	B	C	D	E	f	F	G
EF-Tu_f		11.74	11.30				10.27	10.01
EF-Tu·GTP		11.68	11.59	11.07	10.74 (Asp-21)[b]	10.44	10.27	
EF-Tu·GDP	12.15 (His-85)	11.75 (Gly-95)	11.59	11.06 (Lys-24; Gly)	10.85	10.44	10.26	10.10
EF-Tu·caged GTP	11.94	11.72	11.51	10.97			10.27	
EF-Tu·GMPPNP	11.94	11.72	11.49	11.04			10.28	
EF-Tu·GMPPCP	11.98	11.67	11.46	10.93	10.64			10.17

[a] Peak assignment is according to the EF-Tu·GDP spectrum shown in Figure 2a. The values are given in parts per million downfield from 2,2-dimethyl-2-silapentane-5-sulfonate. [b] The tentative assignment to the amide proton of the corresponding amino acid of *T. thermophilus* is given in parentheses (except for His-85 where it is a ring imido proton); see Discussion.

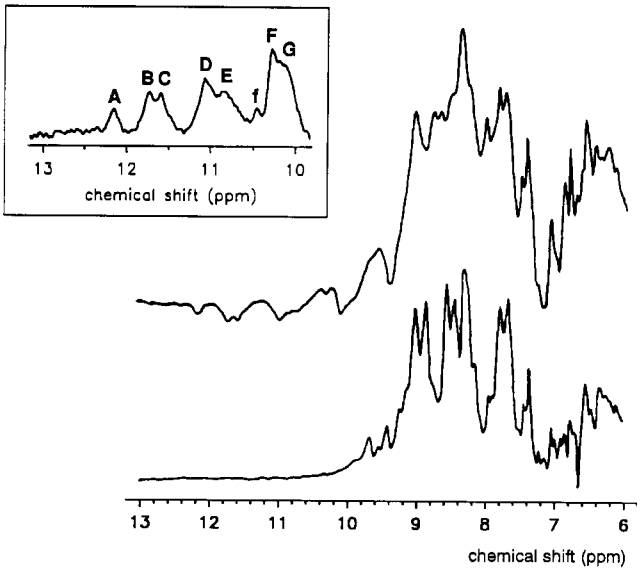

FIGURE 1: 500-MHz proton NMR spectrum of EF-Tu·GDP (upper trace) and domain II and III (lower trace) from *T. thermophilus* at 25 °C. Due to the phase variations inherent in the 1–3–3–1 pulse sequence, the spectral region downfield of about 9.8 ppm has inverted phase with respect to the aromatic part. The phase-corrected downfield region is shown in the insert.

NMR spectra were run on a Bruker AM 500 spectrometer (Karlsruhe, Germany) at a proton resonance frequency of 500 MHz. The strong water signal was suppressed by the 1–3–3–1 pulse sequence (Hore, 1983). The distance between the pulse segments was chosen such as to produce maximum excitation in the spectral region around 11 ppm. Unfortunately, the 1–3–3–1 sequence creates phase variations over the total spectrum width along with frequency-dependent signal intensity alterations. When regarding a limited spectral region, for example, the range between 10 and 13 ppm as in our studies, these changes are of only little influence or can be properly corrected for. Typically between about 2000 and 10000 FID's, depending on protein concentration, have been accumulated to give a reasonable signal-to-noise ratio. Chemical shifts of proton resonances were measured relative to the methyl proton resonance of internal 2,2-dimethyl-2-silapentane-5-sulfonate. The NMR buffer consists of 50 mM borate, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 0.01% (w/v) NaN_3, and 10% (v/v) D_2O. The final protein concentrations of the samples were about 200 μM, and the volume was 500 μL.

Results

Figure 1 shows a 500-MHz proton NMR spectrum for a sample of EF-Tu·GDP from *T. thermophilus* at 25 °C. There are at least eight lines (A–G) in the downfield region below 10 ppm (Table I). We initially assumed that all of these lines are associated with nucleotide binding and arise from protons involved in strong hydrogen bonds formed between phosphate

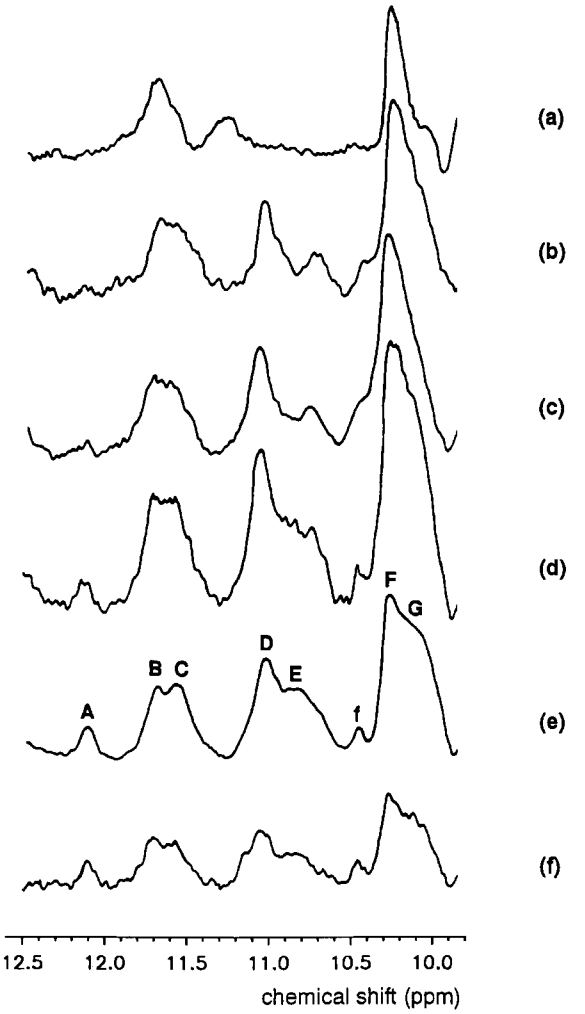

FIGURE 2: Downfield proton NMR spectra of nucleotide-free EF-Tu (a), EF-Tu·GTP (b), and spectra during hydrolysis of protein-bound GTP to GDP (c–e). In spectrum f, a spectrum of EF-Tu·GDP immediately after addition of GDP to EF-Tu_f is shown. Times after addition of nucleotide to EF-Tu_f in minutes are (b) 0 [55], (c) 65 [120], (d) 205 [80], (e) 320 [780], (f) 0 [60]. The time in minutes for acquisition of the spectra is given in brackets.

oxygens of GDP and amino acids of the protein (Redfield & Papastavros, 1990). To verify this assumption, the proton NMR spectrum of the nucleotide-free EF-Tu was studied. In the corresponding spectrum (Figure 2a), three lines (at 11.74, 11.30, and 10.27 ppm) are still present, but several lines seen in the EF-Tu·GDP spectrum are missing. Thus not all of the signals in the downfield region of the spectrum from *T. thermophilus* EF-Tu·GDP can be ascribed to nucleotide binding. These lines are probably due to interdomain or intradomain interactions within the protein.

In order to examine which of the resonances below 10 ppm in the nucleotide-free factor are produced by such interactions,

we measured the NMR spectrum (Figure 1) of the peptide 208–405 (domains II and III) of EF-Tu from *T. thermophilus*, which is unable to bind GTP or GDP (Peter et al., 1990). No resonances below 10 ppm were found in the downfield region of this spectrum (Figure 1). Several lines appear in the upfield region of the spectrum of domain II/III that can be identified with lines also occurring in the spectrum of the intact molecule (Figure 1). As expected, the resolution of this spectrum is better than that for the entire EF-Tu due to the lower molecular weight. This is in accordance with the predictions of Pieper et al. (1990) and Peter et al. (1990), who demonstrated that domain II/III from *E. coli* and *T. thermophilus*, respectively, behaves like an independent folding unit that adopts a secondary and probably tertiary structure similar to that present in the intact EF-Tu.

The EF-Tu_f spectrum (Figure 2a) shows a relatively broad line at 11.3 ppm and a more intense signal at 11.7 ppm. After addition of GTP (Figure 2b) to the nucleotide-free EF-Tu, two lines at approximately 11.7 and 11.6 ppm are observed. During the hydrolysis of bound GTP to GDP (Figure 2c–e), this doublet becomes more distinct. At the same time, an extremely downfield-shifted signal at 12.15 ppm is seen to build up and increase in intensity. With rising temperature, this signal is broadened and shifted upfield and has almost disappeared at 65 °C (Figure 3). This implies that proton exchange with water takes place more rapidly. Significantly, two signals at 11.07 and 10.74 ppm emerge after addition of GTP to EF-Tu_f (Figure 2b). The resonance of about 11 ppm (Table I) can be observed in NMR spectra of all samples of nucleotide-bound EF-Tu (GTP, GDP, GMPPNP, caged GTP). During hydrolysis of the bound GTP to GDP, the line at 10.74 ppm is possibly broadened and shifted downfield. This shift could be explained by an interaction between the now terminal β-oxygen that is more electronegative than the oxygen bridging the β- and γ-phosphate and the amide protons of the protein. In the course of GTP hydrolysis, the formation of an additional small peak at 10.44 ppm is observed whose origin remains unclear.

An alteration of the intensities of the double peak at approximately 11.75 and 11.60 ppm relative to the peaks at 11.06 and 10.85 ppm is observed upon comparing the EF-Tu·GDP spectra at various temperatures (Figure 3). At higher temperatures, the peaks at 10.10 and 10.26 ppm retain their intensities but are separated from each other more distinctly due to a narrowing of the signals. This implies that the protons giving rise to these lines exchange only slowly (on the NMR time scale) with water. This is not surprising since these interactions must be stable enough to maintain the three-dimensional native conformation of this thermostable protein even at higher temperatures. In contrast, the broadening of the line at about 12.15 ppm (25 °C) with increasing temperature is obviously associated with an enhancement of the exchange rate of the corresponding proton with water. Hence it is conceivable that the nucleotide exchange is facilitated at the physiological temperature of about 65 °C. The rates of GDP exchange in the case of EF-Tu·GDP from *T. thermophilus* at 25 and 65 °C, respectively, differ by a factor of 10–13 (unpublished observations).

To prove that the described spectral changes shown in Figure 2 do not reflect a mere adaptation of the EF-Tu conformation to GTP, but rather the hydrolysis of GTP and a concomitant conformation change, we recorded an NMR spectrum of EF-Tu·GDP immediately after addition of GDP to the nucleotide-free factor (Figure 2f). Total agreement between the spectra of the EF-Tu·GDP formed by hydrolysis

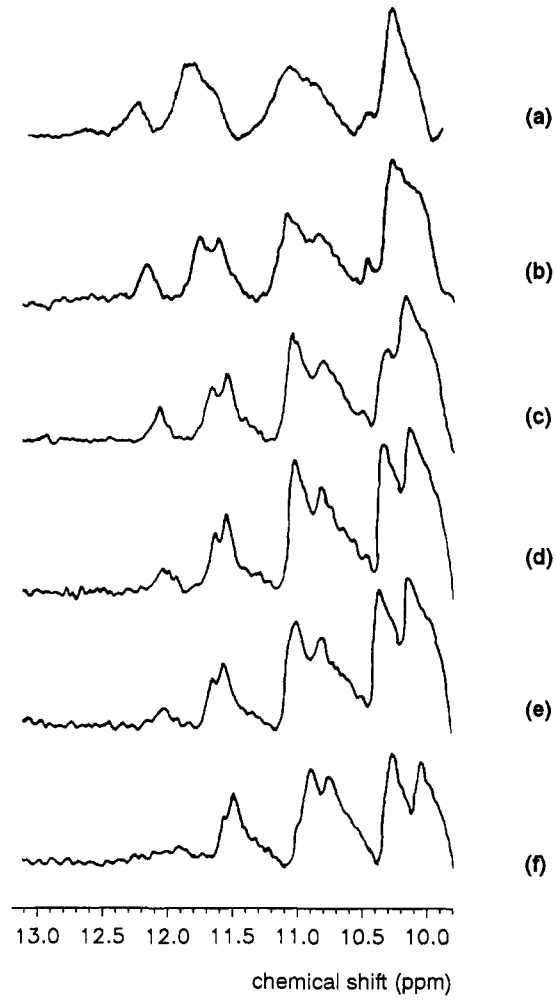

FIGURE 3: Downfield proton NMR spectra of EF-Tu·GDP in dependence on temperature: (a) 4 °C; (b) 25 °C; (c) 35 °C; (d) 45 °C; (e) 55 °C; (f) 65 °C.

from EF-Tu·GTP (17 h at 25 °C, Figure 2e) and the spectrum of EF-Tu·GDP was found. We ascertained that GTP had been completely hydrolyzed to GDP by HPLC analysis after the indicated incubation time at 25 °C. Thus, GTP hydrolysis with the associated conformational change can indeed be inferred from the time course of the NMR spectra. This is clearly demonstrated in Figure 4, where the relative intensity increase (as compared to its relative intensity in EF-Tu·GDP, i.e., after total hydrolysis of bound GTP) of the line at 12.15 ppm is plotted vs time after addition of GTP to nucleotide-free EF-Tu. The data for GTP hydrolysis as determined by HPLC analysis are plotted for comparison purposes. A remarkable agreement between the two experimental values is observed. It is also obvious that GDP binds immediately to EF-Tu_f and that the nucleotide-containing EF-Tu has already adopted the appropriate conformation during the NMR experiment (60 min, 25 °C).

After addition of GMPPNP to the nucleotide-free EF-Tu (Figure 5b), again we observed the resonance signal at about 11 ppm that is also present in all nucleotide-containing EF-Tu spectra. Instead of the poorly resolved doublet at 11.68 ppm, as in the case of EF-Tu·GTP, three well-separated signals can be seen: two sharp peaks at 11.5 and 11.7 ppm and a relatively broad line at 11.9 ppm. The peak at 10.7 ppm of EF-Tu·GTP is missing. In the case of p21·GMPPNP there exists a hydrogen bond between the amide proton of Gly-13 and the NH bridging the β- and γ-phosphate of the nucleotide (Pai et al., 1990). Since this hydrogen bond should be stronger in the

Table II: Partial Alignment of Those Regions in the Protein Sequences of EF-Tu from *T. thermophilus*, *E. coli*, and p21 Being Important for Interpretation of the NMR Spectra

sequence	G	X	X	X	X	G	K	T/S	switch I region									D	X	X	G
EF-Tu *T. thermophilus*	^{18}G	H	V	D	H	G	K	T^{25}	^{57}R	A	R	G	I	T	I	N	T^{65}	^{81}D	C	P	G^{84}
EF-Tu *E. coli*	^{18}G	H	V	D	H	G	K	T^{25}	^{56}K	A	R	G	I	T	I	N	T^{64}	^{80}D	C	P	G^{83}
p21	^{10}G	A	G	G	V	G	K	S^{17}	^{30}D	E	Y	D	P	T	I	E	D^{38}	^{57}D	T	A	G^{60}

sequence	switch II region																	N	K	X	D
EF-Tu *T. thermophilus*	^{84}G	H	A	D	Y	I	K	N	M	I	T	G	A	A	Q	M	D^{100}	^{136}N	K	V	D^{139}
EF-Tu *E. coli*	^{83}G	H	A	D	Y	V	K	N	M	I	T	G	A	A	Q	M	D^{99}	^{135}N	K	C	D^{138}
p21	^{60}G	Q	E	E	Y	S	A	M	R	D	Q	Y	M	R	T	G	E^{76}	^{116}N	K	C	D^{119}

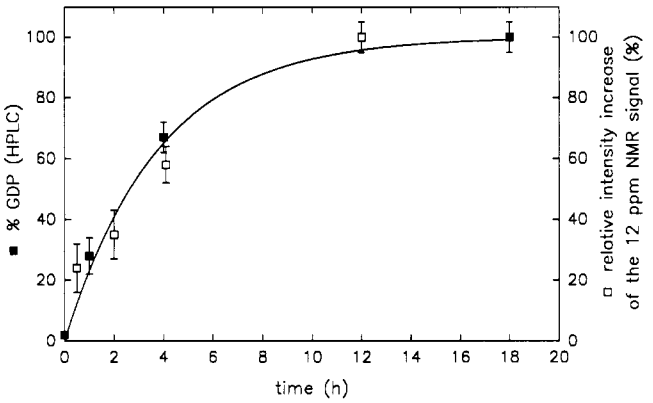

FIGURE 4: Hydrolysis of protein-bound GTP to GDP determined via reverse-phase HPLC analysis (filled squares) on RP-18 (50 mM potassium phosphate, pH 6.7, 10 mM tetrabutylammonium hydrogen sulfate, 5% acetonitrile). The NMR data (open squares) reflect the relative intensity increase of the 12 ppm NMR signal in percent of the signal intensity after total hydrolysis of protein-bound GTP to GDP. The times referring to the NMR data are calculated from the sum of the time after addition of GTP to EF-Tu$_f$ plus half of the NMR measuring time.

case of a phosphodiester with an oxygen than with an imino group between the β- and γ-phosphate, a signal can be observed only in the case of EF-Tu·GTP and not in the case of EF-Tu·GMPPNP. The amino acid corresponding to Gly-13 of p21 is Asp-21 in *T. thermophilus* EF-Tu (Table II). This assignment is also compatible with the results of Lowry et al. (1992), according to which this peak was strongly attenuated after exchange of Mg^{2+} for Mn^{2+} whereas that of Lys-24 was totally suppressed. This suggests a larger distance from the corresponding residue from the metal ion than that of Lys-24. This finding excludes this peak being due to Thr-25 or Thr-62, respectively, which are probably directly coordinated to Mg^{2+} in analogy to the p21 high-resolution X-ray structure data (Pai et al., 1990). The NMR spectrum measured immediately after addition of GMPPNP to the nucleotide-free EF-Tu shows no significant differences to the spectrum of the same sample after 22 h (data not shown), thus excluding the possibility of major conformational changes after binding of the nucleotide taking place on a time scale comparable with GTP hydrolysis rate.

The spectrum of EF-Tu·GMPPCP (Figure 5c) is similar to that of EF-Tu·GMPPNP in the region below 11.2 ppm. The main difference is provided by two broad signals at 10.6 and 10.9 ppm. The latter could correspond to the line at about 11 ppm in all nucleotide-containing EF-Tus. However, we cannot assign the resonance at 10.6 ppm. It may be that new interactions between the nucleotide and EF-Tu are possible due to the larger bond distance between the γ- and β-phosphate resulting from replacement of oxygen by CH_2 as the bridging group, this enabling the γ-phosphate to make additional interactions with the protein.

The binding of caged GTP to EF-Tu becomes visible in the NMR spectrum (Figure 6a) via the appearance of lines which

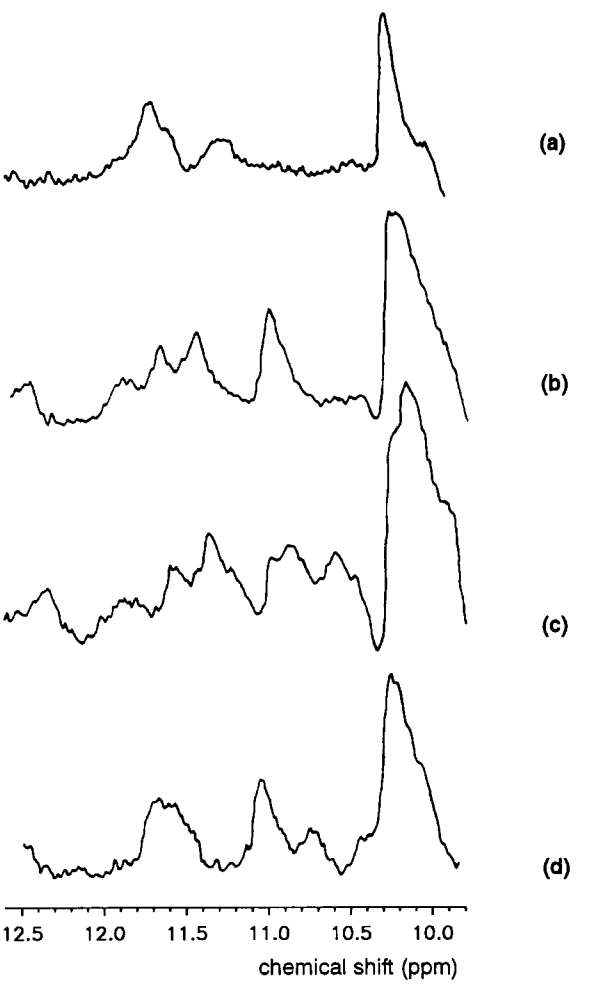

FIGURE 5: Downfield proton NMR spectra of EF-Tu·GMPPNP (b) and EF-Tu·GMPPCP (c). For comparison, the spectra of EF-Tu$_f$ (a) and EF-Tu·GTP (d) are shown.

are also seen after the addition of GTP or GDP to nucleotide-free EF-Tu (Figure 2). Nevertheless, we see differences in the spectra that may reflect a steric hindrance of the aromatic group of caged GTP which prevents the correct binding of the GTP moiety. Again the peak at about 11 ppm is clearly visible. The signal at 10.77 ppm which is detected after addition of GTP to EF-Tu$_f$ is, if it is present at all, strongly broadened. The peak at 11.7 ppm is observed as well. Additionally, there occurs a well-resolved resonance at 11.5 ppm and possibly a less intense broad line at 11.94 ppm.

EF-Tu·caged GTP complex (Figure 6b–d) was photolysed by irradiation at 366 nm for 50 min at 0 °C under conditions of low intrinsic GTPase. We observed the slow buildup of a resonance signal at 12.15 ppm (during the NMR experiment at 25 °C) that became more and more intense with time due to hydrolysis of protein-bound GTP, as in the case of EF-Tu·GTP. Additionally, upfield from a peak at 10.9 ppm, the

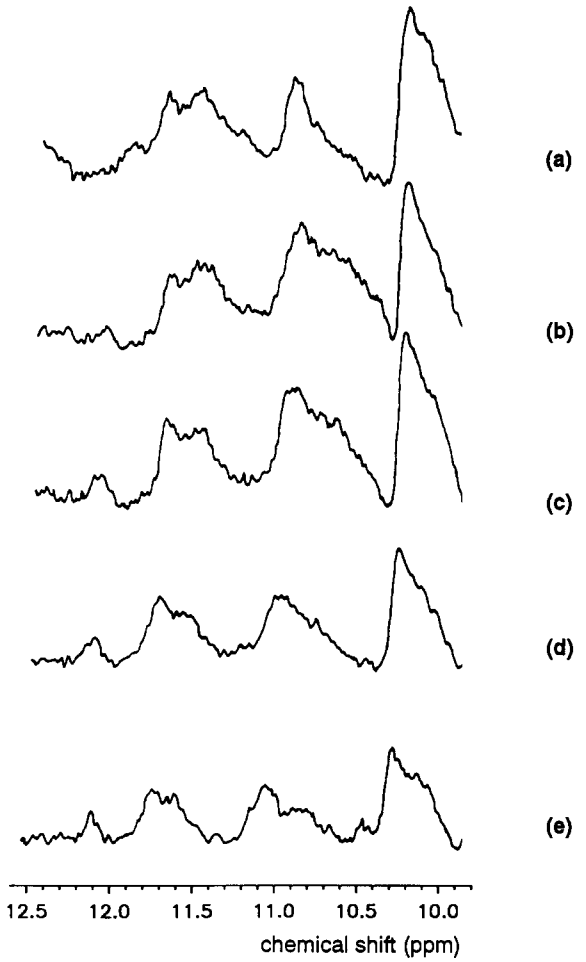

FIGURE 6: Downfield proton NMR spectra of EF-Tu·caged GTP before (a) and after (b–d) photolysis at 366 nm for 50 min at 0 °C. Times after photolysis of caged GTP in minutes are (b) 10 [60], (c) 90 [70], and (d) 230 [120]. The measuring time in minutes is given in brackets. (e) A spectrum of EF-Tu·GDP immediately after addition of nucleotide to $EF\text{-}Tu_f$ is shown.

shoulder at 10.7 ppm is observed. After 6 h at 25 °C, the spectrum (Figure 6d) was identical to that of EF-Tu·GDP (Figure 6e). During this time interval, the GTP was completely hydrolyzed as corroborated by an HPLC control experiment (Figure 7). Interestingly, the spectrum immediately after photolysis of caged GTP is not identical with the EF-Tu·GTP spectrum. It was confirmed, however, by HPLC that after photolysis of the bound caged GTP, GTP was formed but scarcely hydrolyzed. We assume that 2-nitrosophenylmethylketone does not dissociate from the protein and prevents a correct binding of the formed GTP. Likewise, we could not detect the free 2-nitrosophenylmethylketone in the NMR spectra. Only after hydrolysis of the γ-phosphate is the subsequently formed GDP able to interact correctly with the protein.

Discussion

Alignment of the Amino Acid Sequences of p21, EF-Tu from E. coli, and EF-Tu from T. thermophilus. All GTP/GDP-binding proteins contain consensus sequences associated with their nucleotide-binding and functional domains. The high structural homology of EF-Tu and p21 and the high-resolution structure of p21 allow now a more precise alignment of the sequences with respect to their functional domains. Due to their homology, we attempted in this work to assign some resonances in *T. thermophilus* ^1H spectra on the basis of previous p21 NMR investigations (Redfield & Papastavros,

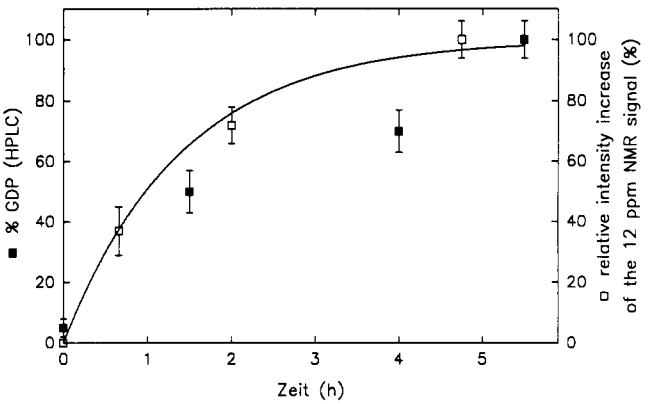

FIGURE 7: Hydrolysis of protein-bound GTP after photolysis of caged GTP analyzed by HPLC (filled squares) and NMR (open squares) are shown. For details, see the legend to Figure 4.

1990). In Table II the alignment of those regions in the protein sequences is given which are important for interpretation of ^1H NMR spectra of *T. thermophilus* EF-Tu.

Tentative Assignment of Downfield Resonances. Nucleotide-free EF-Tu from *T. thermophilus* shows three proton resonances in the downfield region (Table I, Figure 2a). These signals obviously cannot be ascribed to hydrogen bonds between the phosphate groups of the nucleotide and the protein.

Lowry et al. (1992) have measured the ^1H–^{15}N HMQC spectra of ^{15}N-labeled amide groups of glycines in an overproduced *T. thermophilus* EF-Tu·GDP (Ahmadian et al., 1991). They observed two downfield glycine resonances at 11.9 and 11.3 ppm at 35 °C. The isolated G domain of *E. coli* EF-Tu exhibits only one downfield glycine resonance at 11.3 ppm (Lowry et al., 1991). Assuming that the G domain in *T. thermophilus* and *E. coli* EF-Tu have a homologous three-dimensional structure, it is possible that the glycine resonance at about 11.7 ppm for nucleotide-free EF-Tu and for nucleotide-containing EF-Tu results from an interaction of domain I (G domain) and domain III rather than from an intradomain interaction within the G domain. In the model for the entire *E. coli* EF-Tu, Jurnak et al. (1990b) assume that the Gly-83–Gly-100 region of domain I forms intramolecular contacts with the domain III of EF-Tu. Clark et al. (1990) propose that the contact between the G domain and domain III is created between the helices B (residues 85–93) and C (residues 113–125) and parts of the β-strands a (residues 301–311), e (residues 372–378), and f (residues 381–391) of domain III [Figure 2 in Clark et al. (1990)]. In both models it is Gly-94, although it does not belong to helix B in the Clark model, that is in particular proximity to domain III. This amino acid, corresponding to Gly-95 in *T. thermophilus*, is located in a region which is supposed to exhibit conformational differences between EF-Tu·GDP and EF-Tu·GTP (Jurnak et al., 1990b) The downfield shift of this presumed Gly-95 (11.7 ppm) correlates with a strong hydrogen bond. The proton acceptor is possibly a carboxyl oxygen. According to the EF-Tu structure of Clark et al. (1990), it could be possible that this acceptor is provided by the carboxyl group of Glu-378 in *E. coli* (Glu-390 in *T. thermophilus*). Gly-95 lies in a region (residues 84–100) which is clearly analogous to residues 60–76 in p21 (Jurnak et al., 1990a,b). It is part of the "switch II" region whose conformation changes when GDP is replaced by GTP in the p21 molecule (Milburn et al., 1990). We hypothesize that Gly-95 in *T. thermophilus* is a part of the linker which enables a flow of information between the G domain and domain III. It may also transfer long-range effects to domain II during the conformational changes between the

inactive EF-Tu·GDP ("switch off") and the active EF-Tu·GTP ("switch on"). The "switch II" region is believed to be the recognition site for the putative nucleotide exchange factor (Milburn et al., 1990). Although the nucleotide exchange factor, EF-Ts, forms a complex with the isolated domain II/III (Peter et al., 1990), we have shown that for the tight interaction of EF-Ts with EF-Tu the stabilizing effect of the G domain is necessary. EF-Ts has no significant functional effect on the isolated G domain of *E. coli* EF-Tu (Parmeggiani et al., 1987). Contact between the Gly-95 region may propagate conformational changes during receptor binding on domain II/III to domain I and enable nucleotide exchange. We proposed (Peter et al., 1990) that the lack of interaction of isolated domain II/III with aa-tRNA is due to an absence of a long-range effect by which domain I codetermines the structure of domain II/III.

In the region corresponding to the peak D, Lowry et al. (1992) are able to uniquely assign a lysine and a glycine in an ^{15}N HMQC NMR spectrum. We suggest that one of these resonances be ascribed to Lys-24 in agreement with the findings of Lowry et al. (1992b). Up to now it is not possible to uniquely assign the glycine residue. The lysine is part of the invariant GXXXXGK sequence. According to the p21 tertiary structure, this amino acid should in both protein conformations (EF-Tu·GTP and EF-Tu·GDP) be located in the proximity of the β-phosphate of the nucleotide and is able to form hydrogen bonds between its amide proton and the oxygen of the phosphate. According to the p21·GMPPNP structure of Pai et al. (1990), the main-chain amide of Lys-16, corresponding to Lys-24 in *E. coli* and *T. thermophilus*, interacts with the β-phosphate oxygen of the nucleotide. At the momentary stage of crystallographic analysis of EF-Tu from *E. coli*, the invariant Lys-24 seems to interact with the β-phosphate of GDP (Jurnak et al., 1989; Clark et al., 1990) as do the main-chain amides of the GXXXXGK loop.

It cannot totally be exluded that one of the downfield peaks originates from the guanine imino proton which by analogy with p21 (Pai et al., 1990) should form a hydrogen bond with the carboxyl group of Asp-139. However, in that case a resonance should be observed for all EF-Tu–nucleotide complexes nearly at the same position. From our proton NMR studies, it cannot be definitely deduced whether this is true or not. A decision could be made by using ^{15}N-labeled guanosine nucleotide and performing a ^{1}H–^{15}N difference decoupling NMR experiment (Griffey & Redfield, 1987).

The line below 12 ppm in the EF-Tu·GDP spectrum which builds up during hydrolysis of protein-bound GTP arises from a histidine imidazole proton since in the ^{15}N HMQC studies of Lowry et al. (1992) (D. F. Lowry and A. G. Redfield, personal communication) the signals below 12 ppm showed very large ^{15}N chemical shifts characteristic for histidine imidazole protons. We propose to assign this peak to His-85, which very probably plays a crucial role in the hydrolysis of protein-bound GTP, quite analogous to the corresponding Gln-61 residue in p21 (Pai et al., 1990). Similar to the case of serine proteases, the correct tautomeric form of the histidine imidazole ring could be stabilized by an interaction with the carboxyl group of a nearby aspartate or glutamate. The most likely candidates appear to be Asp-87, corresponding to Glu-63 in p21 (Pai et al., 1990), and Glu-118, which is adjacent to His-85 according to the crystal structure data of EF-Tu·GDP from *E. coli* (Clark et al., 1990). However, the role of the serine side chain is presumably played by a water molecule, which then is able to make a nucleophilic attack on the γ-phosphate of GTP. Probably we see in the NMR spectra the protonation of the His-85 imidazole residue by proton transfer from the water molecule. The subsequently positively charged imidazole ring could be stabilized by the negative charge of the carboxyl oxygen of Asp-87 or Glu-118, respectively.

The application of 2-nitrobenzyl caged substrates may cause problems during the investigation of proteins. After photolysis of caged GTP, the released nitrosoketone is able to react with thiol groups, including cysteines and other nucleophilic groups, thereby modifying the protein (Kaplan et al., 1978). A corroboration for this prediction is that we were not able to detect the free 2-nitrosophenylethylketone in the NMR spectra of EF-Tu·caged GTP after photolysis. Interestingly, the only cysteine (Cys-82) in EF-Tu from *T. thermophilus* is part of the DXXG consensus sequence in GTP/GDP-binding proteins, which is involved in phosphate binding. Cys-82 probably reacts with the released photolabel.

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Registry No. GTP, 86-01-1; GDP, 146-91-8; GTPase, 9059-32-9.

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NMR Study of the Phosphate-Binding Loops of *Thermus thermophilus* Elongation Factor Tu†

David F. Lowry,‡,§ Mohammed Reza Ahmadian,‖ Alfred G. Redfield,*,‡ and Mathias Sprinzl‖
Departments of Physics and Biochemistry, Brandeis University, Waltham, Massachussetts 02254, and Laboratorium für Biochemie and Institut für Makromolekulare Forschung, Universität Bayreuth, Postfach 10 12 51, D-8580 Bayreuth, FRG
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ABSTRACT: The phosphoryl-binding loops in the guanosine diphosphate binding domain of elongation factor Tu were studied by ^{15}N heteronuclear proton-observe NMR methods. Five proton resonances were found below 10.5 ppm. One of these was assigned to the amide group of Lys 24, which is a conserved residue in the phosphoryl-binding concensus loop of purine nucleotide binding proteins. The uncharacteristic downfield proton shift is attributed to a strong hydrogen bond with a phosphate oxygen. The amide protons from the homologous lysines in N-ras p21 [Redfield, A. G., & Papastavros, M. Z. (1990) *Biochemistry 29*, 3509-3514] and the catalytic domain of *Escherichia coli* elongation factor Tu [Lowry, D. F., Cool, R. H., Redfield, A. G., & Parmeggiani, A. (1991) *Biochemistry 30*, 10872-10877] also resonate downfield in similar positions. We propose that the downfield shift of this lysine amide proton is a spectral marker for this class of proteins. We also have studied the temperature dependence of the downfield resonances and find a possible conformation change at 40 °C.

There is a large class of proteins that bind and hydrolyze purine nucleoside triphosphates, whose structures change when they hydrolyze purine nucleoside triphosphate to purine nucleoside diphosphate. The energy of hydrolysis is coupled to other processes via the conformational change. For example, myosin hydrolyzes the ATP during muscle contraction, and adenylate kinase hydrolyzes ATP as it phosphorylates AMP. Elongation factor Tu (EFTu)[1] hydrolyzes GTP to GDP after it positions the aminoacyl transfer ribonucleic acid on the ribosomal A site. These proteins bind purine nucleoside triphosphate in similar ways, even though their global structures bear no resemblence to each other. There is ample evidence from X-ray crystallographic studies that the α- and β-phosphate oxygens fit into an anion hole. The hole is formed by the backbone amides of the well known eight-residue consensus loop: Gly,X,X,X,X,Gly,Lys,(Ser or Thr) (henceforth loop 1) (Dreusike & Schulz, 1988; la Cour et al., 1985; Jurnak, 1985; Clark et al., 1990; Wooley & Clark, 1989; Jurnak et al., 1990; Pai et al., 1989, 1990; Milburn et al., 1990: Schlichting et al., 1990, Bourne et al., 1990, 1991).

Saraste et al. (1990) have compared the sequences of loop 1 among several such proteins from different origins, and they found that the lysine residue is the only absolutely conserved residue in this loop, which suggests that it has a conserved structural role. In H-ras p21, adenylate kinase, and EFTu crystal models, the lysine is at the amino terminus of an α-helix, and its amide group points toward the phosphate oxygens. The lysine side chain spans the loop, and the ϵ-amino group hydrogen bonds to the carbonyl oxygen of the first glycine. Thus, both the side-chain amino group and the

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* To whom correspondence should be addressed.
‡ Brandeis University.
§ Present address: Department of Chemistry, University of Oregon.
‖ Universität Bayreuth.

[1] Abbreviations: HMQC, heteronuclear multiple-quantum correlation spectroscopy; EFTu, elongation factor Tu; HSMQC, heteronuclear single-multiple-quantum correlation spectroscopy; MSL, maleimido-PROXY spin-label; G-domain, catalytic domain of *E. coli* EFTu.

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