Characterization of Surface Topology and Binding Area in Complexes of the Elongation Factor Proteins EF-Ts and EF-Tu · GDP from *Thermus thermophilus*: A Study by Protein Chemical Modification and Mass Spectrometry

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Dedicated to Professor Dieter Seebach on the occasion of his 60th birthday

Abstract: Amino acetylation of lysine residues in combination with mass-spectrometric peptide mapping of tryptic peptides has been applied to the characterization of surface topology and binding areas in complexes of the translation–elongation factors EF-Tu and EF-Ts from *Thermus thermophilus*. Electrospray/ionization mass spectrometry (ESI-MS) yielded pH-dependent differences in the charge structure for the molecular ions of EF-Ts indicating differences in solution structure. The noncovalent EF-Tu · GDP complex was directly identified by ESI-MS. Acetylation reactions of EF-Ts, EF-Tu · GDP, and EF-Ts · EF-Tu complexes with varying molar ratios of acetic anhydride were directly followed by mass spectrometry, which identified the precise number of acetyl groups in the partially modified proteins. The acetylation sites and relative reactivities of the lysine residues were determined from mass-spectrometric peptide-mapping data of tryptic peptide mixtures; the modified sites were identified by on-line HPLC–ESI-MS. The comparison of the acetylation pattern for the free proteins with that of the EF-Tu · EF-Ts complex enabled the characterization of structural changes in the effector loop of EF-Tu, around the Lys-45 and Lys-52 residues, upon complex formation. Two domains in EF-Ts, the *N*-terminal region comprising Lys-21 and Lys-45 and a central helix–turn–helix region comprising Lys-119, Lys-133, and Lys-140, were shielded from acylation in the complex; this shows their different accessibilities in free and complexed EF-Ts. These results show the usefulness of an approach combining of differential chemical modification combined with mass spectrometry for probing surface topology and molecular interactions in protein complexes.

Keywords: acylations · mass spectrometry · peptides · protein–protein interactions · supramolecular complexes

Introduction

Elongation factor Tu (EF-Tu) is an essential component for bacterial protein biosynthesis. EF-Tu belongs to the guanosine-5′-triphosphatase (GTPase) superfamily and promotes the binding of aminoacyl tRNA to the mRNA codon-programmed ribosomes. During its functional cycle EF-Tu binds to several ligands: guanosine 5′-triphosphate (GTP), aminoacyl-tRNA, ribosomes, guanosine 5′-diphosphate (GDP), and elongation factor Ts (EF-Ts).[1] A characteristic feature of the elongation cycle is a switch from an inactive (GDP-binding, EF-Tu · GDP) to an active (GTP-binding, EF-Tu · GTP) conformation;[2] the GDP to GTP ligand exchange is promoted by EF-Ts. GDP and GTP are both strongly bound to EF-Tu, with a K_D for EF-Tu · GDP of 10^{-9} M.[1, 3] The EF-Tu · GDP complex from *E. coli* has three domains:[4] domain I (G domain) contains the nucleotide-binding region and consists mainly of α/β bundles, while domains II and III have β-sheet conformation. The crystal structures of the native EF-Tu · GDP complexes from *T. aquaticus* and *E. coli* have been determined only recently[5, 6] and showed that the structurally flexible effector loops form a β-sheet structure. The crystal structure of EF-Tu from *T. thermophilus* is that of a complex with the nonhydrolyzable phosphamido analogue of GTP, guanosine 5′-(β,γ-imido)triphosphate (GPPNHP),[7] and by comparison with EF-Tu · GDP has almost identical subunit structures but dramatic differences in the arrangement of domains upon cleavage of the GTP-γ-phosphate group.[2] In the EF-Tu · GPPNHP complex, the effector loop (also termed switch I region) has an α-helical conformation.[8]

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EF-Ts from *T. thermophilus* has been isolated in vitro as a stable complex with EF-Tu.[9] EF-Ts forms a homodimer by means of an intermolecular disulfide bond. The stoichiometry of the complex can be described as EF-Tu · $(EF\text{-}Ts)_2$ · EF-Tu.[10] The crystal structure of an *N*-terminal-truncated, inactive EF-Ts from *T. thermophilus*[11] showed substantial differences to EF-Ts from *E. coli*. This could be an explanation for the lack of binding of the *E. coli* EF-Ts to EF-Tu from *T. thermophilus*.[9] The X-ray structure of the (EF-Tu · $EF\text{-}Ts)_2$ complex from *E. coli* has been recently reported.[12] However, molecular interaction of EF-Tu · GDP with EF-Ts from *T. thermophilus* is still unknown.

The combination of selective chemical modification of the teriary structure and mass-spectrometric peptide mapping has been developed as an efficient approach for the molecular characterization of specific modification sites and their relative reactivities.[13, 14] This method has already found several applications in studies of protein structure and structure – function relationships.[15–25] The general analytical scheme (Figure 1) comprises the mass-spectrometric determination of the precise number of modified groups and their distribution as a first step, followed by identification of the modified sites and their relative reactivities by mass-spectrometric peptide mapping.[22] Several amino acid-specific modification reactions have been found feasible, such as amino acylation, tyrosine iodination or nitration, and the bifunctional cysteine modification by phenylarsonous acid derivatives.[14, 23, 24] The efficiency of this approach has been recently illustrated by selective succinylation at the inner channel surface of a porin ion-channel protein, which selectively modified ion transport and selectivity.[25] In this study we have characterized surface topology and binding areas of the EF-Tu · EF-Ts complex from *T. thermophilus* by lysine acetylation combined with mass-spectrometric peptide mapping. The differential amino acylation and mass-spectrometric analysis enabled us not only to characterize the binding area of subunits, but also to identify structural changes upon complex formation and shielding effects in different regions of the complex.

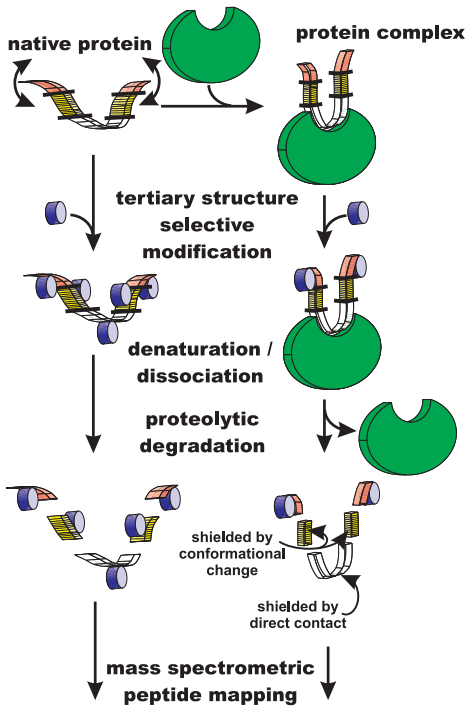

Figure 1. Analytical scheme for the characterization of protein tertiary structures and noncovalent complexes by selective chemical modification and mass spectrometry. Modification groups and proteolytic cleavage sites are represented by filled circles and lines, respectively.

Results

ESI mass spectrometry (ESI-MS) and conformational characterization of elongation factors: The elongation factor proteins were characterized by ESI-MS determination of molecular weights and distributions of multiply charged molecular ions. The molecular weights determined were 44632 Da for EF-Tu and 44555 Da for the EF-Ts disulfide-linked dimer (Table 1). These data are precisely consistent with the posttranslational removal of *N*-terminal methionine residues from both proteins,[11, 26] which was confirmed by mass-spectrometric peptide mapping as shown below.

ESI mass spectra of proteins display continuous series of multiply charged macromolecular ions with charge states and distributions (charge structures) characteristic of solution structural states.[27, 28] An increase of charge states with increasing pH was observed for EF-Ts. At pH 5 the charged 24+ molecular ion ($[M+24\,H]^{24+}$) was most abundant, whereas at pH 7 a bimodal distribution of molecular ions was observed with the $[M+31\,H]^{31+}$ ion being most abundant (Figure 2a). These changes of charge structures cannot be explained by solvent parameters such as protonation strength, which would result in the opposite effect, that is increased

Table 1. MALDI-mass-spectrometric molecular weight determinations of EF-Ts, EF-Tu, and their acetylated derivatives.

	Molar excess anhydride	M_r (obsd)	M_r (calcd)[a]	No. acetyl groups	Extent of modification[b]
EF-Ts[c]	–	44555	44557	–	–
	10	45130	45145	14	37
	50	45236	45229	16	42
	100	45433	45439	21	55
EF-Tu · GDP[d]	–	44632	44636	–	–
	10	45033	45013	9	43
	50	46027	46021	23	105
	100	46280	46273	29	132

[a] A mass increment of 42 Da per acetyl group was used to calculate the molecular weight of the acetylated derivatives. [b] Values are given in %. [c] EF-Ts forms a covalently linked homodimer. [d] GDP dissociated from the EF-Tu · GDP complex prior to MALDI-MS analyses in unmodified and modified EF-Tu · GDP with 10-fold molar excess acetic anhydride.

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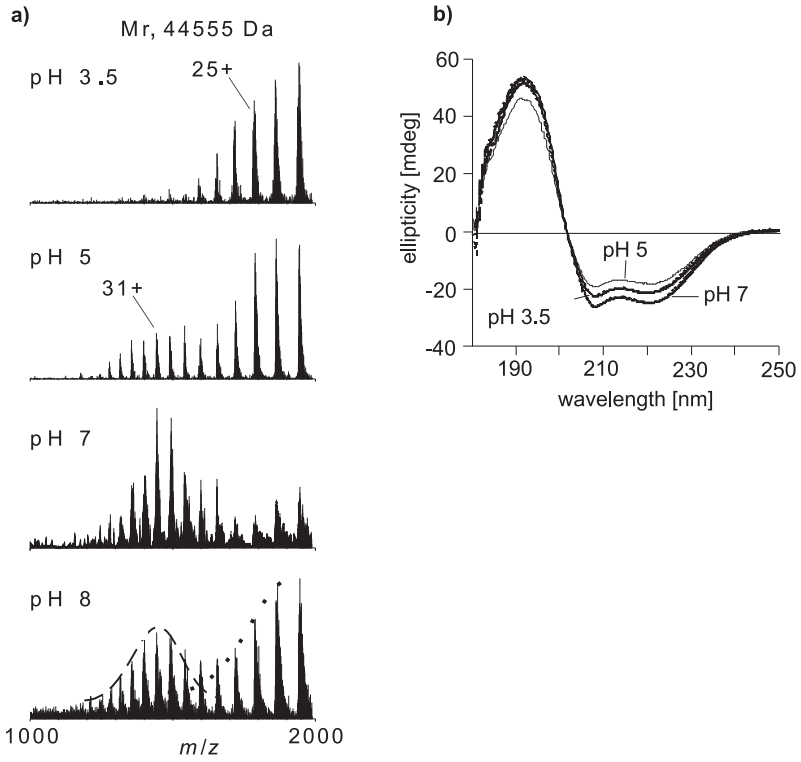

Figure 2. pH-Dependent ESI-MS and CD spectra of EF-Ts. a) ESI-mass spectra of EF-Ts in 2 mM NH_4OAc/methanol (9:1, v/v); pH was adjusted by addition of ammonia. Charge numbers denote $[M+nH]^{n+}$ ions; the molecular weight determined is 44555 Da (M_r calcd: 44557 Da). Conformational states suggested by the charge state distributions are indicated by dotted lines. b) pH-Dependent CD spectra of EF-Ts in 2 mM NH_4OAc.

charge states with decreasing pH. Instead, they are consistent with conformational changes of the protein.[28–33] No pH-dependent conformational change of EF-Ts was observed in circular dichroism (CD) spectra (Figure 2b), which revealed a predominantly α-helical conformation. Hence, the ESI-MS and CD data suggest a structural change of EF-Ts such as reorientation of two or more protein domains relative to each other that does not affect secondary structure elements. Since native EF-Ts occurs as a dimer, the charge structures of ions may also reflect different orientations of the monomer units relative to each other.

The use of ESI-MS for the direct analysis of noncovalent interactions has been employed in several recent studies of supramolecular protein complexes.[27, 28, 34–36] The ESI spectrum of the EF-Tu · GDP complex at pH 3 was comparable with that of the nucleotide-free EF-Tu, showing a homogeneous series of ions around the most abundant $[M+35H]^{35+}$ ion for the apo-protein only (Figure 3). In contrast, at pH 6 the EF-Tu · GDP complex is represented by the series of additional ions (Figure 3, inset); under these conditions (tenfold molar excess of GDP) the molecular weight corresponds exactly to an EF-Tu · 2 GDP complex was determined, indicating the coordination of a second GDP probably to the binding site of the 3′ end of tRNA.

Characterization of amino acetylated EF-Tu and EF-Ts derivatives: EF-Tu · GDP and EF-Ts were acetylated at constant pH (7.5) with varying molar ratio of acetic anhydride. The average number of acetyl groups introduced was directly determined by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and ESI-MS. Both methods consistently yielded increasing molecular weights for the acetylated protein derivatives with increasing excess of anhydride (Table 1, MALDI-MS data). ESI spectra of EF-Tu and EF-Ts derivatives acetylated to a small extent showed charge structures of molecular ions nearly identical to the unmodified proteins (data not shown), indicating that the native structure is retained. A gradual increase of acylation was found for up to approximately 50% (Table 1) of the total number of amino groups in the EF-Ts dimer (36 lysine residues and 2 *N*-termini). The selective amino acetylation of EF-Ts was ascertained by identification of all the modified sites as shown below.

The acetylation of the EF-Tu · GDP complex afforded a similar gradual increase in molecular weights. However, at 50- and 100-fold molar excess of acetic anhydride the molecular weight increase (1385 Da and 1644 Da, respectively) considerably exceeded the molecular weight increment corresponding to the total number of amino groups present (21 lysine-ε-amino groups

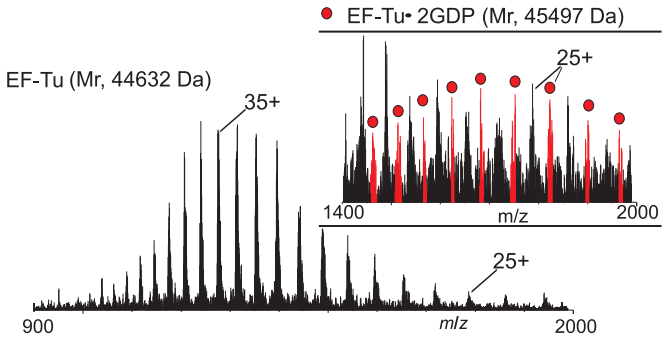

Figure 3. ESI mass spectra of EF-Tu and the EF-Tu · 2 GDP complex. The ion series, centered around the $[M+35H]^{35+}$ ion signal at pH 3 (2% HOAc/methanol 9:1), yields a molecular weight of 44632 Da (M_r calcd: 44636 Da). The spectrum (inset) obtained at pH 6 (2 mM NH_4OAc/methanol 9:1) shows an additional ion series due to the EF-Tu · 2 GDP complex (M_r 45497 Da, indicated by the filled circles).

and an *N*-terminus). Although such a result may, in principle, be caused by additional O-acylation, quantitative acetylation at serine and tyrosine residues has been shown to occur only under strongly acidic reaction conditions.[37] The subsequent peptide-mapping results clearly showed that the modified sites were predominantly *N*-acetylated (see below). A possible explanation for the observed excessive increase in molecular weight would be that upon acetylation the GDP cofactor remained in the protein and can only be removed by subsequent denaturation and proteolytic digestion. Indeed, if the molecular weight of GDP is subtracted from that of the

protein derivative, a molecular weight corresponding to acetylation of all the amino groups is obtained at a 50-fold molar excess of acetic anhydride (Table 1). Reversible acetylation influencing cofactor binding has been reported for a cytochrome b5 – heme complex.[38]

Mass-spectrometric identification of acetylation sites and relative reactivities of lysine residues: The sites of *N*-acetylations were identified by peptide-mapping analyses of the protein derivatives: i) by MALDI-MS and ESI-MS after tryptic digestion in solution and ii) by on-line HPLC – ESI-MS of acetylated tryptic peptides (Figure 4). Identification of the

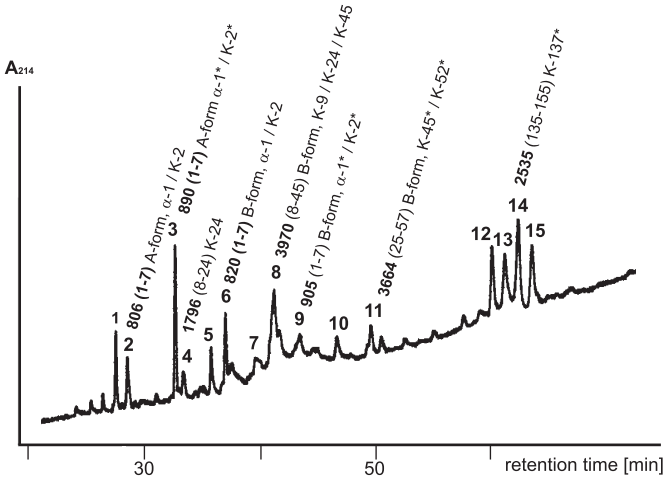

Figure 4. HPLC analysis of a tryptic peptide mixture from partially acetylated EF-Tu. The EF-Tu · GDP complex was acetylated with a 10-fold molar excess of acetic anhydride:NH_2 group, digested with trypsin, and subjected to narrow-bore reversed-phase HPLC. The eluate was monitored by UV (214 nm) and then analyzed by on-line ESI-MS. Peptide sequences and molar masses are assigned above the HPLC peaks in bold print. Acetylated Lys residues are denoted by asterisks.

tryptic peptide mixtures of EF-Ts and their acetylated derivatives, as well as the proteolytic fragments of the EF-Tu · EF-Ts complex, provided almost complete sequence coverage for EF-Ts. For EF-Tu, acetylated tryptic peptides were found predominantly in the G domain. The EF-Tu · GDP complex was found to be highly resistant to proteolytic cleavage in aqueous buffer solutions; therefore tryptic digestion was carried out with addition of 30% methanol.[21] Under these conditions mainly α-helical parts of the protein become accessible to cleavage, whereas β-sheet structures are preferentially denatured by addition of chaotropes such as urea.[21, 25] The peptide-mapping analyses of EF-Tu · GDP also enabled us to indentify mixtures of proteolytic peptides for both the A and B forms of EF-Tu, which are products of different Tu/A and Tu/B genes, respectively.[26] After tryptic digestion, the peptides were analyzed by MALDI-MS peptide mapping and by LC – ESI-MS, which yielded consistent results. As an example, the *N*-terminal peptide 1 – 7 (Figure 4, peak 2) of the A form has a molecular ion at $m/z = 806$ (Val at position 6). The same peptide of the B form (Ile at position 6) has a molecular ion at $m/z = 820$ (Figure 4, peak 6). Another ion doublet with a mass difference of 16 Da occurs as a result of the substitution of two amino acids in the peptide sequence 25 – 57. The A form contains Tyr-33 and Ala-35 residues and thus has an ion at $m/z = 3565$, whereas the B form contains Phe and Thr residues at these positions yielding an ion at $m/z = 3581$. Since these three alternative positions, together with a fourth heterogeneity Arg-264 vs Lys-264 in the A and B forms, respectively, yield identical molecular weights for both forms, structural differentiation is only possible by the mass-spectrometric peptide-mapping data. Peptide 25 – 57 was found to be acetylated at Lys-45 and Lys-52 ($m/z = 3664$) even at a low (10-fold) molar excess of acetic anhydride (Figure 4, peak 11); this indicates the high reactivity of these residues in the EF-Tu · GDP complex.

The acetylation pattern in the EF-Tu · GDP complex provides information about the nucleotide-binding Lys residues. The Lys-137 residue, which is part of the nucleotide-binding consensus sequence -Asn-Lys-Xxx-Asp-, was shown to be acetylated from the shift of the ion representing peptide 135 – 155 to $m/z = 2533$ (observed at $m/z = 2491$ in the unmodified protein, Figure 4, peaks 10 and 14). This result is consistent with X-ray crystallographic data[7] showing that this Lys residue is involved in a lipophilic interaction with the nucleotide by means of its hydrocarbon side chain leaving a freely accessible ε-amino group. In contrast, Lys-24, which is involved in nucleotide binding through its ε-amino group, remained unacetylated. The corresponding tryptic peptide 8 – 24 was identified in all protein derivatives (Figure 4, peak 4).

In addition to these specific *N*-acetylations, residues other than Lys in the G domain of the EF-Tu · GDP complex (Cys-82, Tyr-47, and Tyr-70) were acetylated in the presence of a high molar excess of acetic anhydride. The corresponding peptide ions were observed by MALDI-MS peptide mapping with low abundances (data not shown). Independent analyses of the tryptic peptide mixtures of EF-Ts and their acetylated derivatives by MALDI-MS and ESI-MS produced consistent results and almost quantitative sequence identification. Only the partial sequences -Ala-Asp-Arg-Glu-Ala-Arg- (50 – 55) and -Phe-Glu-Leu-Gly-Ala- (191 – 195, the *C*-terminal peptide) were not observed. An $[M+2H]^{2+}$ ion was observed for the unmodified peptide 11 – 21 at $m/z = 556$ together with the signal for the acetylated peptide 11 – 22 at $m/z = 657$ (Figure 5). As trypsin did not cleave at the acetylated Lys-21 residue, the identification of this modification site is unambiguous (Lys-21 is shielded in the EF-Tu · EF-Ts complex as shown below). Furthermore, an $[M+3H]^{3+}$ ion signal at $m/z = 537$ was found for the unmodified peptide 106 – 119 cleaved by trypsin at Lys-119. Upon acetylation, a corresponding ion signal ($[M+3H]^{3+}$) for the Lys-119-acetylated peptide 106 – 121 at $m/z = 646$ was observed (Figure 5). These results are consistent with MALDI-MS peptide-mapping analysis in which ions for peptide 106 – 119 at $m/z = 1609$, peptide 106 – 121 at $m/z = 1894$, and for the acetylated derivative of peptide 106 – 121 at $m/z = 1935$ were detected as singly protonated ions (Figure 6a). From the corresponding peptide-mapping data, the Lys residues from EF-Ts were categorized according to their relative chemical reactivities (Table 2). These results are in agreement with the surface accessibilities determined by X-ray crystallography of EF-Ts.[14] The amino groups with the highest relative reactivities (Lys-7, Lys-8, Lys-21, Lys-119,

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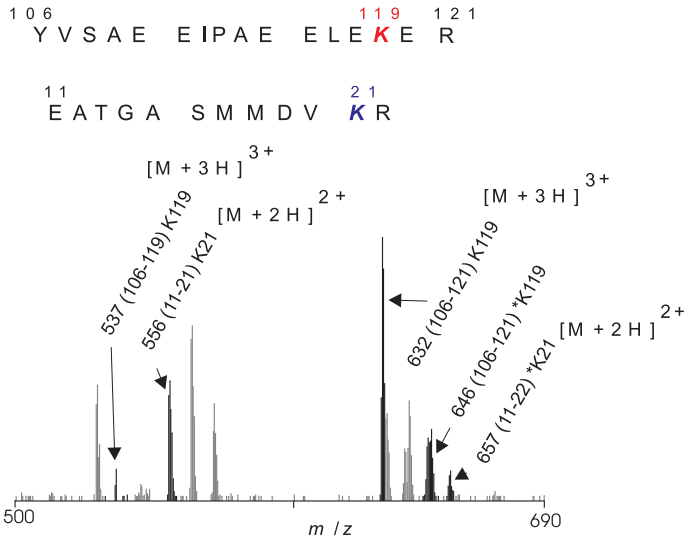

Figure 5. ESI-MS analysis of a tryptic peptide mixture from EF-Ts partially acetylated with a 10-fold molar excess of acetic anhydride:NH_2 group and digested with trypsin. Only $[M+2H]^{2+}$ and $[M+3H]^{3+}$ ions of partial sequences encompassing (11–22) and (106–121) are denoted. Partial sequences are shown on top.

and Lys-133) were those that underwent the most rapid acetylation (Table 2). The corresponding ions of modified peptides were found with increasing abundance at higher (50-fold and 100-fold) reagent excess. In contrast, low reactivities were observed for the *C*-terminal Lys residues (Lys-165, Lys-167, Lys-169, and Lys-178) as evident from the unmodified tryptic peptide 166–178 at $m/z =$ 2345, observed even after reaction with a 100-fold molar excess of acetic anhydride (not shown). Also, Lys-48 was unmodified in all experiments.

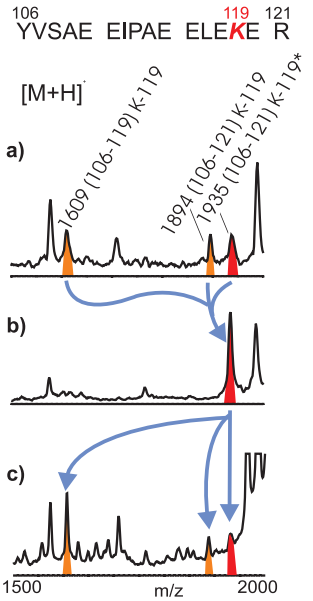

Figure 6. MALDI-MS peptide-mapping analyses of tryptic peptides from EF-Ts and the EF-Tu · EF-Ts complex, partial EF-Ts sequence (106–121). a) Acetylated EF-Ts with 10-fold excess anhydride:NH_2 group. b) Acetylated EF-Ts with 50-fold excess anhydride:NH_2 group. c) Acetylated EF-Tu · EF-Ts complex with 100-fold excess anhydride:NH_2 group. The acetylated peptide is denoted by an asterisk. Spectra were recorded with HCCA as matrix.

Determination of shielded lysine residues in the EF-Tu · EF-Ts complex: Acetylations of the EF-Tu · EF-Ts complex were carried out with 50-fold and 100-fold molar excess of acetic anhydride under conditions previously established to modify all accessible Lys residues in the free proteins. Therefore, reactive Lys residues that were not acetylated in the complex could be characterized as shielded

Table 2. Relative reactivities of lysine amino groups from EF-Ts.

Relative reactivity[a]	Lysine residue[b]
1	α, 7, 8, 21, 119, 133,
2	33, 45, 93,
3	49, 140, 147, 148, 162,
4	48, 165, 167, 169, 178.

[a] Descending reactivity. [b] Lysine residues were identified by MALDI and ESI-MS peptide mapping.

as a result of complex formation. As an example, acetylation of the EF-Tu · EF-Ts complex with a 100-fold molar excess of acetic anhydride produced peptides containing both unmodified and modified Lys-119 (Figure 6c); this indicates that Lys-119 was partially shielded in the complex. In contrast, MALDI-MS peptide mapping of the acetylated free EF-Ts showed substantial modification of the Lys-119 residue at only a tenfold molar excess of acetic anhydride, and complete acetylation of the peptide 106–121 at a 50-fold molar excess of acetic anhydride (Figures 6a and 6b). Comparison of the complete acetylation pattern for free EF-Ts with that of the EF-Tu · EF-Ts complex showed that Lys residues Lys-21, Lys-45, Lys-119, Lys-133, and Lys-140 in EF-Ts were shielded by the interaction with EF-Tu (Table 3). The partial acetylation and tryptic digestion of the EF-Tu · ET-Ts complex produced a complex mixture of modified peptide fragments that

Table 3. Mass assignments of selected lysine containing tryptic peptides from EF-Ts and acetylated derivatives before and after complexation with EF-Tu.

Peptide/ Lys residue	$[M+H]^+$ (calcd)	$[M+H]^+$ (obsd) EF-Ts[a]	EF-Ts · EF-Tu[b]	Acetylated Lys residues
11–21/21	1109	–	1109	–
11–22/21	1266	–	1267	–
11–22/21	1308	1311	–	21
40–45/45	692	–	694	–
40–48/45	963	965	963	–
40–48/45	1004	1004	–	45
106–119/119	1607	–	1607	–
106–121/119	1893	–	1894	–
106–121/119	1935	1936	1934	119
122–145/133, 140	2712	–	2713	133 or 140
122–145/133, 140	2754	2753	2755	133 and 140

[a] 50-Fold molar excess acetic anhydride:NH_2 group. [b] 100-Fold molar excess acetic anhydride: NH_2 group.

included several partial sequences with closely spaced lysine residues that could not be directly assigned from the mass-spectrometric peptide-mapping data. Therefore, on-line LC–ESI-MS analyses were additionally carried out; this resulted in unequivocal peptide identification and assignment of acetylations. For example, in the partial sequence of EF-Ts bearing the three Lys residues Lys-45, Lys-48, and Lys-49 acetylation at Lys-45 and Lys-49 was ascertained by the monoacetylated peptide 40–48 ($m/z = 1004$) that was cleaved by trypsin at the unmodified Lys-48 residue.

The comparison of the acetylation pattern for EF-Tu · GDP with that of the EF-Tu · EF-Ts complex showed that lysine residues Lys-45 and Lys-52 in EF-Tu were effectively shielded by the interaction with EF-Ts (Table 4 and Figure 7). Tryptic digestion of unmodified EF-Tu · GDP produced peptides

Table 4. Mass assignments of selected lysine-containing tryptic peptides from EF-Tu and acetylated derivatives before and after complexation with EF-Ts.

Peptide/ Lys residue	$[M+H]^+$ (calcd)	$[M+H]^+$(obsd) EF-Tu[a]	EF-Ts · EF-Tu[b]	Acetylated Lys residues
8–24/24	1796	1795	1796	–
25–57/45, 52	3664	3664	–	45 and 52
46–57/52	1408	–	1408	–
135–155[c]/137	2490	2491	2491	–
135–155[c]/137	2532	2532	2533	137

[a] 50-Fold molar excess acetic anhydride:NH_2 group. [b] 100-Fold molar excess acetic anhydride:NH_2 group. [c] Cleavage after Phe-134 as a result of the intrinsic chymotryptic activity of trypsin.[21]

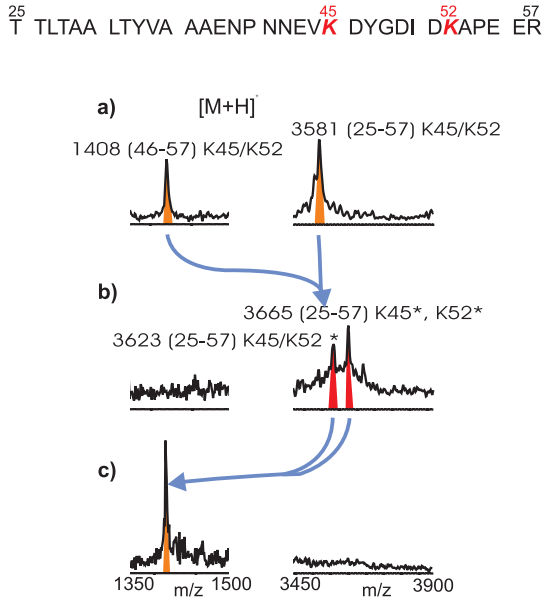

Figure 7. MALDI-MS peptide-mapping analyses of tryptic peptides from EF-Tu and the EF-Tu · EF-Ts complex, partial EF-Tu sequence (25–57). a) Unmodified EF-Tu · GDP. b) Acetylated EF-Tu · GDP with 50-fold excess anhydride:NH_2-group. c) Acetylated EF-Tu · EF-Ts complex with 100-fold excess anhydride:NH_2-group. Acetylated peptides are denoted by asterisks. The ion in c) is the unmodified (shielded) fragment (46–57). Spectra were recorded with HCCA as matrix.

46–57 and 25–57 ($m/z = 3581$, B form) that contained Lys-45 and Lys-52 (Figure 7a). At 50-fold molar excess of acetic anhydride, ion peaks from the nonacetylated peptides disappeared and only ions of the acetylated peptides 25–57 with one and two acetyl groups ($m/z = 3623$ and 3665, respectively) were observed (Figure 7b). In contrast, acetylation of the EF-Tu · EF-Ts complex, even at a 100-fold molar excess of anhydride, yielded exclusively the unmodified peptide 46–57; hence, tryptic cleavage at Lys-45 was used to identify this unmodified residue (Figure 7c). In contrast to these shielding effects for amino groups, other lysine residues in both EF-Tu and EF-Ts showed no differences in their modification pattern. This is illustrated by the ESI spectra from tryptic peptide mixtures for peptide 135–155 from EF-Tu · GDP (Table 4). The unmodified peptide ion $[M+3H]^{3+}$ at $m/z =$ 831 shifted almost completely to the Lys-137-acetylated peptide ion at $m/z = 846$ upon acetylation. An identical distribution of unmodified and Lys-137-acetylated peptide ions was found for the acetylated EF-Tu · EF-Ts complex.

Discussion

The most useful analytical feature of ESI-MS is the direct determination of molecular weights and modification patterns of protein derivatives, with the particular advantage of ESI for characterizing conformational states of native and partially modified proteins.[14] For example, recent pH-dependent ESI-MS studies provided evidence for a correlation of structural states in solution with charge distributions (charge structure) of multiple-protonated molecular ions.[23] Protein ions retain defined conformations in the gas phase that are stable for hours and can be probed by H/D-exchange reactions[39] and ion-mobility techniques.[40, 41] These studies also show that interconversion of protein conformations in the gas phase requires additional energy input.[42] For EF-Ts, the observed pH dependence of the charge distribution is consistent with the dynamic interchange of tertiary structures in solution.[43]

Several applications of ESI-MS for the characterization of noncovalent protein complexes with low molecular-weight ligands such as substrates or inhibitors have been reported.[28, 34–36] In the present study, ions corresponding to the EF-Tu · 2 GDP complex were detected; this is in agreement with the specific binding of one GDP molecule in the nucleotide-binding pocket of the G domain and uptake of a second GDP molecule probably in the aminoacyl-adenosine-binding cleft.[23] These results suggest that ESI-MS can be used successfully for the molecular characterization of protein–nucleotide interactions in conjunction with protein-chemical and spectroscopic methods for their structural characterization.

The comparative mass-spectrometric analysis of protein derivatives obtained by differential chemical modification is shown to be a powerful approach for probing tertiary structure differences.[13, 14] In a recent example, X-ray crystallography in combination with mass spectrometry was successfully applied to an ion-channel protein, after selective succinylation of lysine residues, where intact tertiary structures and specific ion conductances were maintained in the protein derivatives;[25] hence, this approach seemed applicable also to the study noncovalent complexes in their native forms. In the present study, mass-spectrometric peptide mapping has been used for the direct assignment of modification sites and relative reactivities from the amino acetylated derivatives of EF-Tu · GDP, EF-Ts, and the noncovalent EF-Tu · EF-Ts complex. This technique also enabled us to identify particular regions that were shielded as a result of the complex formation. The characteristic proteolytic pattern shown here, that is, the acylation of lysine residues that become inaccessible to trypsin digestion, can be readily extended to other site-specific modifications such as amidation of carboxylate groups that afford Glu and Asp residues that cannot be cleaved by the *S. Aureus* V8-protease.[20] Mass-spectrometric peptide mapping is found to be particularly efficient for the characterization of initially modified proteins, which are often significant for structure–reactivity relations.[44]

Generally, acetylation of an accessible lysine ε-amino group in a protein ($P-NH_2$) competes with the complex formation of P with a binding protein (Q) that results in the shielding of this particular amino group. The shielded amino group ($P-NH_2$) may either be involved in specific binding or in other

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contacts in the $P-NH_2 \cdot Q$ complex. The competing reactions are shown in Equation (1).

$$[P-NH-Ac]+[Q] \underset{k_r[Ac]}{\overset{k_{-2}}{\rightleftarrows}} [P-NH_2]+[Q] \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} [P-NH_2 \cdot Q] \quad (1)$$

For the stable EF-Tu · EF-Ts complex, k_{on} (8.3×10^6 M^{-1}s^{-1}) is large in comparison with k_{off} (0.17 s^{-1}) as determined by fluorescence quenching.[45] Therefore, two situations may be considered: i) $k_2 \ll k_{on}$; in this case virtually no acetylation will be observed for amino groups shielded by complex formation; ii) $k_2 \gg k_{on}$ and also therefore $k_2 \gg k_{off}$; here, acetylation of amino groups buried in the complex will occur according to the complex dissociation (on the presumption that concentration of acetylating agent, [Ac], is very high and concentration dependence of the reaction can be neglected). Since the amino acetylation is essentially irreversible under the present conditions, k_2 is much larger than k_{-2} (e.g., $k_2 =$ 19.3 M^{-1}s^{-1} for succinylation of aniline[46]). In the EF-Tu · EF-Ts complex, k_2 is much smaller than k_{on} so that the acetylation kinetics should follow i). Our results are in good agreement with these considerations, although the above rate constants were determined under slightly different conditions than applied here and do not take into account partial protonation of lysine ε-amino groups ($pK_a \approx 11$) under neutral conditions and the simultaneous hydrolysis of acetic anhydride in aqueous solutions. However, despite competing hydrolysis, the final degree of modification of an accessible amino group is a function of the amount of reagent and the ratio of the amino acetylation vs hydrolysis rates.[47] As the hydrolysis of acid anhydrides is slow relative to the amino acetylation (e.g., $k_{HOH} = 2.7 \times 10^{-3}$ s^{-1} for acetic anhydride[48]), high yields of acetylated lysine residues are obtained, enabling us to accurately determine the shielding effects in protein complexes.

The present results and those of previous studies[14] ascertained the potential of mass-spectrometric peptide mapping for probing the surface topology of amino groups in tertiary structures. A direct correlation of the present chemical reactivity data with the surface structure of the *T. thermophilus* EF-Ts is not possible owing to the lack of the *N*-terminal region in EF-Ts used for the X-ray structure determination. However, structural information obtained from the mass-spectrometric data is consistent with the recently published X-ray structure of the EF-Tu · EF-Ts complex from *T. thermophilus*,[49] which was obtained after the completion of the present study.[43] Our results show that the Lys-45 and Lys-52 residues in EF-Tu, which are part of the effector loop,[5, 6, 8] become shielded towards amino acetylation upon complexation with EF-Ts. Whether this shielding is cause by direct contact between both proteins or by local structural changes in the flexible EF-Tu region cannot be unequivocally determined from the mass-spectrometric data and the X-ray analysis does not provide structural information about this extremely mobile element of EF-Tu.[49] However, a local structural change of this flexible region in EF-Tu upon complex formation is in agreement with the kinetics of the tryptic cleavage at Arg-59 in the EF-Tu · EF-Ts complex.[50]

A structural comparison of the EF-Ts monomer from *E. coli*[12] and *T. thermophilus*[11] shows that amino acids in the *N*-terminal part are highly conserved, indicating the functional importance of this domain. This has been confirmed by the complete loss of function upon proteolytic removal of the *N*-terminal part of EF-Ts.[10] By contrast, proteolytic cleavage of the *N*-terminus of EF-Ts is prevented in the EF-Tu · EF-Ts complex, a fact indicating its direct contact to EF-Tu.[51] Direct interactions of Lys-23 and Lys-51 residues from EF-Ts by salt bridges to Asp-141 and Asp-21 residues from EF-Tu have been shown in the X-ray structure of the *E. coli* EF-Tu · EF-Ts complex,[12] in agreement with the crystal structure of the *T. thermophilus* complex.[49] The mass-spectrometric data show that the corresponding residues Lys-21 and Lys-45 in *T. thermophilus* EF-Ts become shielded in the EF-Tu · EF-Ts complex, and confirm that binding of EF-Tu by the *N*-terminal part of EF-Ts in *T. thermophilus* is very similar to that in *E. coli*. In contrast to the above described shielding effects, acetylation of Lys-7 of EF-Ts from *T. thermophilus* was observed, as this residue interacts with EF-Tu through its alkyl side chain without involving the ε-amino group.

Three further lysine residues (Lys-119, Lys-133, and Lys-140) in EF-Ts that were found to be partially shielded from acetylation are not involved with direct interactions with EF-Tu. The shielding from acetylation of these residues is therefore caused by a conformational change of EF-Ts upon binding to EF-Tu. The interaction sites and shielding effects of the relevant lysine residues of EF-Ts and EF-Tu by the complex formation are schematically illustrated in Figure 8.

These results clearly indicate the value of protein chemical modification and mass spectrometry to provide structural information complementary to X-ray crystallography; the application of this approach to tRNA · EF-Tu complexes appears promising and will be subject of further studies.

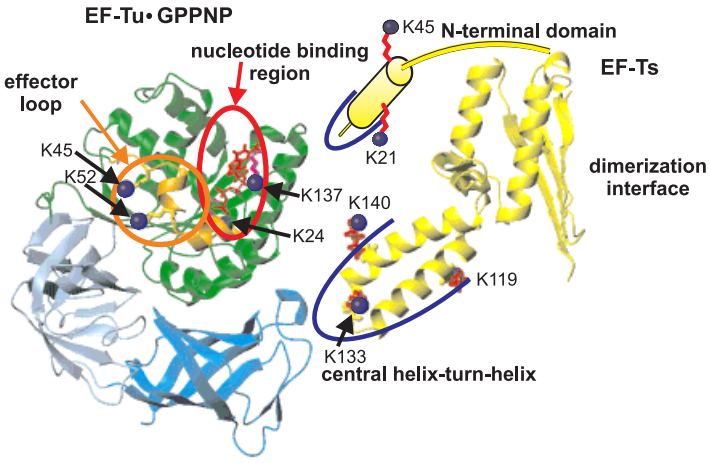

Figure 8. Schematic representations of structures and interacting regions of EF-Tu · GPPNHP and truncated EF-Ts from *T. thermophilus*. The ribbon diagrams show the locations of relevant lysine residues in EF-Tu and one EF-Ts monomer by means of the X-ray coordinates.[7, 11] Lys-45 and Lys-52, which are shielded from acetylation by the EF-Ts interaction, and Lys-24 and Lys-137, which are involved in nucleotide binding, are denoted in the EF-Tu structure. Lys-21 and Lys-45 denoted in the *N*-terminal domain of EF-Ts are shielded by direct interaction. Lysine residues Lys-119, Lys-133, and Lys-140 in the central helix-turn-helix domain are partially shielded by complexation. The structure of EF-Ts shown is derived from the crystal structure of *N*-terminally truncated EF-Ts fragment,[7] the *N*-terminal domain of EF-Ts is depicted schematically (see the complete crystal structure[49]). This figure was created with the MOLMOL software.[56]

Experimental Section

Expression and purification of EF-Tu · GDP: *T. thermophilus* HB8 cells were grown to the mid-log phase in a modified Castenholz medium at 73 °C.[52] Cultures were harvested 3 h after inoculation. Cell lysis and purification of EF-Tu · GDP involving anion-exchange chromatography on Q-Sepharaose Fast Flow [Pharmacia, Uppsala (Sweden)] and gel filtration on Sephacryl S-200 (Pharmacia) were performed as previously described.[53] For final purification of EF-Tu · GDP a further anion-exchange chromatography was performed. The Sephacryl S-200 pool containing EF-Tu · GDP was loaded onto a Mono Q column (0.5 × 5 cm, Pharmacia), equilibrated with α,α,α-tris(hydroxymethyl)-methylamine (Tris)/HCl (50 mM, pH 7.5), KCl (50 mM), and $MgCl_2$ (10 mM), and then eluted with a linear gradient of KCl in Tris/HCl (50 – 500 mM, pH 7.5). Fractions were monitored for EF-Tu activity by SDS/PAGE. Fractions containing EF-Tu · GDP were pooled, dialyzed against Tris/HCl (50 mM, pH 7.5), KCl (50 mM), $MgCl_2$ (10 mM), 2-mercaptoethanol (1 mM), and glycerol (50 %), and stored at −20 °C. The purity of EF-Tu · GDP was estimated by SDS/PAGE to be greater than 98 %. Aliquots were dialyzed against acetic acid (2 %, pH 3) and ammonium acetate (2 mM, pH 6), and were assayed by ESI-MS and MALDI-MS and for EF-Tu activity.

Overexpression and purification of EF-Ts: The EF-Ts-overproducing *E. coli* strain BL21(DE3)pETTs7 was grown as previously reported.[54] The cells were disrupted and the lysate was heat-denaturated in order to precipitate most of the *E. coli* proteins. The recombinant EF-Ts was purified from the supernatant by anion-exchange chromatography on DEAE cellulose DE52 [Whatman, Maidstone (UK)] and subsequent cation-exchange chromatography on EMD-SO_3^- 650 Tentagel [Merck, Darmstadt (Germany)] as previously described.[54] The fractions with EF-Ts activity were pooled, dialyzed against Tris/HCl (50 mM, pH 7.5), KCl (50 mM), and glycerol (50 %), and stored at −20 °C. The purity of EF-Ts was estimated by SDS/PAGE to be > 98 %. Aliquots were dialyzed against acetic acid (2 %, pH 3) and assayed by ESI-MS and MALDI-MS.

EF-Tu activity assay: The EF-Tu · GDP assay[55] was carried out either in Tris/HCl (50 mM, 100 μL, pH 7.5) or in ammonium acetate (2 mM, pH 7). Both solutions contained NH_4Cl (150 mM), $MgCl_2$ (10 mM), 2-mercaptoethanol (5 mM), [^{3}H]GDP (5 μM, 100 mCi mmol^{-1}; Amersham-Buchler), and bis(trimethylsilyl)acetamide (BSA, 1 mg mL^{-1}). After addition of EF-Tu · GDP up to a final concentration of 1 μM, the sample was incubated for 10 min at 37 °C. The reaction was stopped by addition of 1 mL of cold buffer consisting of Tris/HCl (10 mM, pH 7.5), $MgCl_2$ (10 mM), and NH_4Cl (10 mM). The mixture was adsorbed on a nitrocellulose filter [Sartorius, Göttingen (Germany)]. The filter was washed with 1 mL of the same buffer and the retained radioactivity on the dried filter was counted. Activity was comparable for both solvent systems. One unit corresponds to the amount of protein that binds 1 pmol GDP.

EF-Ts activity assay: EF-Ts catalyzes the GDP exchange of EF-Tu-bound GDP. The filter-binding assay[55] measures the exchange of GDP by EF-Tu · GDP (1 μM) at 0 °C for 1 min in Tris/HCl (50 mM, 100 μL, pH 7.5) containing $MgCl_2$ (10 mM), NH_4Cl (150 mM), 2-mercaptoethanol (5 mM), [^{3}H]GDP (5 μM, 100 mCi mmol^{-1}), and BSA (1 mg mL^{-1}). The reaction was started by addition of EF-Ts and stopped by addition of 1 mL cold buffer containing Tris/HCl (10 mM, pH 7.5), $MgCl_2$ (10 mM), and NH_4Cl (10 mM), followed by filtration through nitrocellulose (Sartorius). The filter was washed with 1 mL of the same buffer, and the retained radioactivity on the dried filter was counted. One unit of EF-Ts catalyzes the exchange of 1 pmol EF-Tu-bound GDP in 1 min at 0 °C.

Complex formation and preparation of aminoacetylated EF-Tu · GDP, EF-Ts, and EF-Tu · EF-Ts derivatives: The EF-Tu · EF-Ts complex was formed by incubation of EF-Tu · GDP (25 μM) and EF-Ts (25 μM) in Tris/HCl (50 mM, pH 7.5) for 10 min at room temperature. Monomers were removed by filtration with a Centricon MWCO 100 [100 kDa cut-off membrane, Amicon, Beverly (USA)] at 15 °C. Final protein concentration was 100 μM. The solution was diluted 4 times with Tris/HCl (50 mM, pH 7.5) and filtration was repeated. The purified EF-Tu · EF-Ts complex, EF-Tu · GDP, and EF-Ts were treated with acetic anhydride as previously described.[14] Acetylation of lysine and *N*-terminal amino groups was performed with varying concentrations of acetic anhydride (10, 50, 100 mol per mole of amino groups) in Tris/HCl (50 mM, pH 7.5). Reactions were carried out at constant pH 7.5 (maintained by addition of 25 % NH_3) for 10 min at 20 °C. Products were dialyzed against NH_4HCO_3 (10 mM, pH 7.8) and assayed by ESI-MS and MALDI-MS or subjected to proteolysis.

Proteolytic digestion of EF-Tu · GDP, EF-Ts, and EF-Tu · EF-Ts complex: Digestion of unmodified and aminoacetylated EF-Tu · GDP, EF-Ts, and EF-Tu · EF-Ts complex was performed with trypsin [EC 3.4.21.4, Sigma, St. Louis (USA)] in NH_4HCO_3 (50 mM) containing methanol (30 %) at 37 °C for 3 h with a substrate-to-trypsin ratio of 100:1.[21] Aliquots of the obtained peptide mixture were analyzed by MALDI-MS without further purification or were subjected to on-line LC – ESI-MS.

CD-instrumentation and acquisition conditions: CD spectra were recorded on a JASCO-J-600 CD spectropolarimeter [Jasco, Gross-Umstadt (Germany)] with solutions of EF-Ts (100 μM) in ammonium acetate (10 mM, pH 3.5, 5, and 7). A solvent spectrum was subtracted from 6 averaged original spectra and the molar ellipticity was determined. A scan range of 185 – 250 nm was selected and the scan speed was adjusted to 1 nm min^{-1}.

ESI-MS instrumentation and acquisition conditions: ESI-MS was performed with a Vestec-201A quadrupole mass spectrometer [Vestec, Houston, Texas (USA)] equipped with a thermally assisted electrospray interface. The spray interface temperature was approximately 40 °C for all measurements. The mass analyzer with a nominal *m/z* range of 2000 was operated at unit resolution. An electrospray voltage at the tip of the stainless steel capillary needle of 2 – 2.2 kV and repeller voltages of typically 10 – 50 V were employed. Spectra were recorded with a scan rate of 7 s scan^{-1} with a mass window of $m/z = 200 – 2000$. Mass calibration was performed with the 8+, 9+, and 10+ ions of hen eggwhite lysozyme, and raw data analyzed by means of a Teknivent Vector 2 data system [Teknivent, Houston, Texas (USA)]. Protein and peptide solutions were pumped with a Harvard microinfusion pump [Harvard, Franklin (USA)] through a fused silica capillary (inside diameter, 75 μm) with a flow rate of 2 μL min^{-1} into the ion source. Samples were diluted to a final concentration of 0.1 mg mL^{-1} with acetic acid (2 %, pH 3)/methanol (9:1, v/v) and NH_4OAc (2 mM)/methanol (9:1, v/v). pH was adjusted to 4 – 8 with NH_4OH (25 %).

On-line LC – ESI-MS of tryptic peptides: The microbore HPLC system consisted of an Applied Biosystems 140B solvent delivery system equipped with an Applied Biosystems 759A absorbance detector [Foster City, CA (USA)] set at 214 nm. The tryptically digested peptide mixtures from acetylated EF-Ts, EF-Tu · GDP, and EF-Tu · EF-Ts were separated by means of a microbore C18-column (Sperisorb, 100 × 1 mm, 3 μm). Samples were injected by means of a Rheodyne injection port (model 8125) [Cotati, CA (USA)] equipped with a 5 μL sample loop. Solvent A was trifluoroacetic acid (TFA) in H_2O (0.1 %), and solvent B was TFA in acetonitrile (0.07 %). The flow rate was adjusted to 40 μL min^{-1}. After sample injection the solvent mixture was kept constant at 5 % of B for 5 min and was then altered to 70 % of B over a time period of 60 min. ESI-MS was performed on a Perkin Elmer Sciex API-III triple quadrupole mass spectrometer [Thornhill, Ontario (Canada)] in the single quadrupole scanning mode.

MALDI-MS instrumentation and acquisition conditions: MALDI-MS analyses were carried out with a Bruker Biflex linear time-of-flight spectrometer [Bruker – Franzen, Bremen (Germany)] equipped with a UV nitrogen laser (337 nm) and a dual microchannel plate detector. For the molecular mass determinations the acceleration voltage was set to 25 kV, and spectra were calibrated with cytochrome *c* or myoglobin as internal and external standards. For peptide-mapping experiments the acceleration voltage was set to 10 kV, and insulin and neurotensin were used for internal and external mass calibration. Samples were diluted 10-fold with CH_3OH/0.1 % TFA (2:1, v/v), and 1 μL of this solution was mixed with 1 μL of saturated α-cyano-4-hydroxy cinnamic acid (HCCA) solution dissolved in CH_3OH/0.1 % TFA (2:1, v/v). Spectra were recorded after evaporation of the solvent and processed by means of the X-MASS data system.

Protein structure examination: The structures of EF-Tu · GPPNHP and truncated EF-Ts were examined with files containing the corresponding X-ray coordinates were obtained from Berchtold et al. (1993)[7] and Jiang et al. (1996),[11] on a Silicon Graphics workstation with the Biosym Insight II/Discover and Biopolymer software.[56]

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