Manipulation of the *tuf* gene provides clues to the localization of sequence element(s) involved in the thermal stability of *Thermotoga maritima* elongation factor Tu

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Truncated versions of the *tuf* gene for elongation factor Tu (EF-Tu; 400 aa) from the hyperthermophilic bacterium *Thermotoga maritima* have been produced by progressive 3' → 5' trimming. The truncated genes have been expressed in *Escherichia coli* and the thermal stability of the gene products has been assayed by monitoring their GDP-binding capacity after preheating the cell-free extracts at various temperatures (65–95 °C). One of the truncated proteins, corresponding to the nucleotide-binding domain (G domain; aa 1–200) appears to be only slightly less stable than the full-length EF-Tu. Replacement of the first 90 N-terminal residues of both the full-length *Thermotoga* EF-Tu and the isolated G domain with the corresponding sequence of the mesophilic bacterium *E. coli*, drastically destabilizes both the complete and the truncated protein, indicating that sequence element(s) that are crucial for the attainment of a thermally stable conformation of the *Thermotoga* EF-Tu lie well within the initial portion of the G domain between residues 1 and 90. The relevant residues defy identification, however, as no amino acid preferences, or exclusive sequence element(s), appear to distinguish the N-terminal region of the thermophilic proteins from those of mesophilic counterparts. It is suggested that the thermal stability of *Thermotoga* EF-Tu is critically dependent upon unique tertiary structural interactions involving certain N-terminal residues of the molecule.

Keywords: elongation factor Tu, extreme thermophiles, thermostability, protein engineering, genetic manipulation

INTRODUCTION

Proteins from hyperthermophilic micro-organisms maintain their heat resistance upon expression in mesophilic hosts (Love & Streiff, 1987; Tiboni *et al.*, 1989; Sanangelantoni *et al.*, 1992), indicating that heat stability is solely due to intrinsic features of the molecule rather than to extrinsic factors (Fabry *et al.*, 1989; Schultes *et al.*, 1990). Nonetheless, no unique amino acid substitution scheme, or exclusive sequence motif, distinguishes the thermophile proteins from their mesophile counterparts (Adams, 1993). This implies that thermal resistance may be brought about by subtle (and elusive) primary structural changes resulting in increased hydrophobicity, chain rigidity and

overall tightness of intramolecular packing (Nosoh & Sekiguchi, 1990). However, evidence for this is scanty.

The elongation factors ubiquitously involved in amino-acyl-tRNA binding to ribosomes (EF-Tu for Bacteria; EF-1a for Archaea and Eucarya) offer a good case in protein stability studies. First, the available sequences (about 400 relatively conserved residues) span a host of evolutionarily diverse organisms including the hyperthermophilic bacterium *Thermotoga maritima* (Bachleitner *et al.*, 1989) and an as yet unpublished sequence of *Aquifex pyrophilus* (W. Ludwig, Technische Universität München, Germany, personal communication). Secondly, the stability difference between the *T. maritima* factor, which withstands temperatures up to 90–95 °C, and that of the mesophile *E. coli*, which is inactivated at temperatures slightly above 50 °C, is far greater than that seen for most other proteins. Thirdly, the *T. maritima* gene for EF-Tu

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(*tuf*) can be overexpressed in *E. coli* and the heterologous gene product can be easily purified via the selective denaturation of the (heat-labile) host-cell proteins (Tiboni *et al.*, 1989). Lastly, the EF-Tu N-terminal domain involved in GTP binding and hydrolysis (about 200 residues) retains its GTP-binding capacity following removal of the middle and C-terminal domains involved in the binding of aminoacyl-tRNA, elongation factor Ts and kirromycin-like antibiotics (Weijland *et al.*, 1992). In principle this enables one to selectively modify *tuf* regions corresponding to individual EF-Tu domains and to assess the stability of the encoded protein by means of simple functional assays. This approach may thus allow the localization of sequence element(s) that are responsible for the increased overall stability of the thermophile protein.

In this report, truncated versions of the *T. maritima* EF-Tu, and chimeric (mesophile/thermophile) versions of both the full-length protein and the G domain, have been generated by manipulation of the *tuf* gene; the modified gene sequences have been expressed in *E. coli* and the thermal stability of the gene products has been monitored by means of GDP-binding assays. The results suggest that sequence element(s) located in the N-terminal portion of the G domain play a crucial role in maintaining the thermally stable conformation of the *T. maritima* protein, although no distinguishing primary structural features can be identified through comparison of thermophile and mesophile EF-Tus.

METHODS

Bacterial strains and plasmids. Plasmids pTM6 (Tiboni *et al.*, 1989) and AB80 (Bernardi & Bernardi, 1979), containing the *tuf* genes of *T. maritima* and *E. coli*, respectively, were used to prepare the truncated and chimeric genes. Plasmids pDR720 (Russell & Bennet, 1982), pBR322 (Bolivar *et al.*, 1977) and pUC19 (Vieira & Messing, 1982) were used as the vectors and *E. coli* HB101 as the host. Plasmid-containing strains were grown either in LB medium or in M9 medium supplemented with 50 µg ampicillin ml⁻¹.

Recombinant DNA techniques. Plasmid DNA was isolated and purified using Qiagen tips following the procedures suggested by the supplier (Diagen). Recovery of DNA fragments from low-melting-point agarose gels was performed according to Higuchi *et al.* (1981). Transformation experiments were carried out as described by Dagert & Ehrlich (1979). Truncated *tuf* genes were obtained using the progressive deletion system 'Erase-a-Base' (Promega) following the manufacturer's instructions. The 3' end of the inserts cloned in pUC19 was sequenced by the dideoxynucleotide chain-termination method of Sanger *et al.* (1977). DNA was radiolabelled with [α -³⁵S]dATP α S (Amersham; sp. act. > 37 TBq mmol⁻¹) and primed as specified by the manufacturer using a T7 sequencing kit (Pharmacia).

Plasmid constructions. The recombinant plasmid pTM6 was digested with *Sal*I. The DNA fragment of 1905 bp, containing the entire *tuf* gene and the 3' end of the *fus* gene, encoding the elongation factor G, was recovered from low-melting-point agarose gel and ligated into the unique *Sal*I site of the expression vector pDR720 under the control of the inducible *trp* promoter. The *T. maritima* insert, together with the inducible *trp* promoter, was then transferred to the *Eco*RI site of pUC19 and the plasmid pTu1 was obtained (see Fig. 3). The control plasmid pTu6,

containing the *trp* promoter and the 3' end of the *fus* gene, was derived from plasmid pTu1 by complete deletion of the EF-Tu-coding sequence using the 'Erase-a-Base' system. This plasmid was also used as the vector to construct the recombinant plasmid pTuB. The pTuB plasmid, harbouring the *E. coli tufB* gene under the *trp* promoter, was produced by *Eco*RV digestion of the recombinant plasmid AB80 followed by cloning of the insert (about 3500 bp) into the *Eco*RV site of pBR322. From this plasmid, the *tufB* gene was first subcloned in pUC19, and then excised by digestion with *Bam*HI and *Sph*I (pUC19 polylinker sites) and inserted in the control plasmid pTu6 digested with the same restriction enzymes (see Fig. 3).

Induction of transformed *E. coli* cells. *E. coli* HB101 cells harbouring plasmids containing either the *T. maritima tuf* gene, or the manipulated genes, were grown at 37 °C to OD₆₅₀ 0.5 in M9 salt medium supplemented with 1.0 mM magnesium sulphate, 0.2% glucose and 0.3% Casamino acids; 3- β -indoleacrylic acid was then added to a concentration of 5.0 µg ml⁻¹ and the incubation was continued for a further 2.5 h. Cells were collected by centrifugation at 5000 *g* for 10 min and washed with buffer A (50 mM Tris/HCl, pH 8.0, 10 mM MgCl₂, 50 mM KCl, 7.0 mM 2-mercaptoethanol) and stored at -20 °C until use.

Preparation of cell-free extracts and GDP-binding assays.

Cells were broken by grinding with twice their own mass of alumina powder. The paste was extracted with buffer A (3.0 ml per g wet cells). After centrifugation at 30000 *g* for 30 min to remove alumina and cell debris, the clear extracts were centrifuged at 100000 *g* for 2 h and 100 µl aliquots of the resultant ribosome-free supernatant (S100, containing 8–12 mg protein ml⁻¹, depending on the preparation), were incubated for 10 min at various temperatures between 65 °C and 95 °C. As noted elsewhere (Tiboni *et al.*, 1989), a preliminary 10 min incubation of the S100 at 65 °C is essential for the activation of the *T. maritima* EF-Tu expressed by transformed *E. coli* cells and for denaturation of most *E. coli* host-cell proteins.

After removal of precipitated proteins (10 min centrifugation at 10000 *g*) the GDP-binding activity was assayed by measuring the amount of [³H]GDP (Amersham; sp. act. 459 GBq mmol⁻¹) bound by 10 µl of the heat-treated extracts, during a 10 min incubation at 30 or 65 °C (Tiboni *et al.*, 1978).

Separation of chimeric G domain. All operations were carried out at 4 °C. Cell-free extracts from 15 g of cells were prepared in buffer B (50 mM Tris/HCl, pH 7.4, 10 mM MgCl₂, 80 mM KCl, 7.0 mM 2-mercaptoethanol, 15%, v/v, glycerol) as described previously. The protein solution (35 ml) was applied to a 2.5 × 35 cm DEAE-cellulose DE52 column equilibrated with buffer B. After adsorption of the sample, the column was developed with 1 l 80–320 mM KCl gradient in buffer B at a constant flow rate of 50 ml h⁻¹ while collecting fractions of 12 ml. Fractions were assayed for GDP-binding activity at 30 °C and the protein profile was determined by SDS-PAGE. Positive fractions were pooled, dialysed against buffer B without glycerol, and solid ammonium sulphate was added to 75% saturation. After centrifugation at 20000 *g* for 15 min the protein precipitate was dissolved in buffer B containing 10 mM GDP.

PAGE. Proteins were analysed by SDS-PAGE (Laemmli, 1970). The following markers were used: lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), BSA (66.2 kDa) and phosphorylase b (92.5 kDa). Proteins were stained with Coomassie brilliant blue.

Sequence analysis. Analysis of the DNA sequence was performed using the DNA STRIDER 1.0 program (Marck, 1988).

EF-Tu homologous sequences were retrieved from the databases using the BLAST program (Altschul *et al.*, 1990) through the Human Genome Mapping Project (HGMP) facility, Cambridge, UK. Multiple alignments were obtained using the CLUSTAL W (1.4) program in the GCG software package.

RESULTS AND DISCUSSION

The *T. maritima* EF-Tu sequence was aligned with all of the bacterial and archaeal sequences presently available in the databanks, and with an as yet unpublished EF-Tu sequence from the hyperthermophilic bacterium *Aquifex pyrophilus* (courtesy of W. Ludwig) probably representing the deepest known branching in the bacterial tree (Burggraf *et al.*, 1992; Bocchetta *et al.*, 1995). For concision of presentation only two mesophilic and two thermophilic sequences are shown in Fig. 1 (a global alignment of the *T. maritima* and *Thermus* EF-Tus with all mesophilic counterparts is available from the authors upon request). All of the bacterial sequences appear to be highly conserved (65.2–86.8% overall sequence identity), especially within the N-terminal region harbouring the three consensus elements (GXXXXGK, DXXG and NKXD; underlined in Fig. 1) that are involved in the formation of the GTP-binding pocket (Dever *et al.*, 1987). Unlike the situation with other thermophile proteins (Argos *et al.*, 1979; Fabry *et al.*, 1989; Schultes *et al.*, 1990) analysis of the thermophile EF-Tu sequences showed no

obvious trend towards replacement of small amino acids with bulkier and more hydrophobic ones; also, careful inspection of alignment positions failed to reveal primary structural elements exclusive to the three thermophile sequences. Furthermore, amino acids shared only by the two hyperthermophilic sequences (*T. maritima* and *Aquifex*) were detected at a limited number of sites. Nevertheless, their involvement in thermal resistance can be ruled out by comparison with the corresponding archaeal sequences. For instance, a T to S substitution at position 26 of the alignment (indicated by a vertical arrow in Fig. 1) is found in both the *T. maritima* and the *Aquifex* sequences; its importance for the thermal stability of the two (eu)bacterial EF-Tus is dubious, however, as an S residue at the corresponding sequence position is evident in all of the available archaeal sequences (from both mesophilic and thermophilic sources). This prevented site-directed mutagenesis experiments aimed at identifying single residues that could be responsible for the high stability of the thermophile protein. Consequently, the sequence element(s) involved in the thermal stability of *T. maritima* EF-Tu were investigated by constructing progressively deleted, or chimeric, *tuf* genes, and testing the heat resistance of the resultant gene products.

Inspection of the restriction map of the plasmid pTu1, containing the *T. maritima tuf* gene, revealed a unique *Bgl*II site situated 263 bp upstream from the termination codon of *tuf*, which created ends compatible with those produced by the *Bam*HI site of the pUC19 polylinker. Accordingly, a first deletion clone (termed pTu2) was produced by digestion of pTu1 with restriction enzymes *Bgl*II and *Bam*HI followed by ligation (Fig. 1). Additional progressive deletions were produced by the 'Erase-a-Base' system starting from a *Bgl*II/*Kpn*I-digested pTu1 plasmid. The resultant recombinant plasmids were analysed for insert length and only inserts having a suitable size were sequenced at the 3' end. Clones having a termination codon immediately downstream of the *T. maritima tuf* sequence, namely pTu3, pTu4, pTu5 (whose encoded polypeptide is indicated by an arrow on the last amino acid in the alignment shown in Fig. 1) and pTu6 (in which the entire *tuf* gene had been deleted), were selected for further analysis.

Expression of the truncated genes and thermal stability of the gene products

As Fig. 1 shows, all of the polypeptides encoded by the truncated *tuf* genes contain the three consensus elements required to form the nucleotide-binding pocket. Therefore, the presence of the expressed proteins could be monitored by measuring the GDP-binding capacity of the cell-free extracts at 65 °C, a temperature treatment that allows offsetting of host-cell EF-Tu activity and is also required to activate the entire (overexpressed) *T. maritima* EF-Tu (Tiboni *et al.*, 1989). In essence, cell-free extracts were prepared from *E. coli* HB101 cells transformed with plasmids harbouring the entire *T. maritima tuf* gene (plasmid pTu1), or the progressively deleted versions thereof (plasmids pTu2 to pTu5), and comparable extracts

Fig. 1. Alignment of the *T. maritima* (Tm) EF-Tu amino acid sequence with the sequences of EF-Tu from: Tt, *Thermus thermophilus* (Seidler *et al.*, 1987); Ec, *E. coli*; and Sp, *Spirulina platensis* (Buttarelli *et al.*, 1989). The three consensus elements are underlined. The amino acid sequence of the products of progressively deleted genes is indicated by an arrow on the last amino acid. A vertical arrow also indicates the S residue present in both the *Thermotoga* and *Aquifex* sequences. An asterisk indicates the position of the *Bcl*I site. Residues identical to the *Thermotoga* sequence are indicated by dots.

Fig. 2. Expression in *E. coli* of progressively deleted *Thermotoga tuf* genes. (a) SDS-PAGE analysis on 10 µl aliquots of the extracts of *E. coli* transformed with the following plasmids: pTu6 (control), pTu1 (harbouring the gene encoding the *T. maritima* EF-Tu), pTu2, pTu3 and pTu4 (encoding the isolated G domain), heated at the indicated temperatures (°C). NT, Not treated. The gel electrophoretic patterns are representative of all S100 preparations obtained. Identical gel patterns and similar proportions of heterologous proteins were observed in different preparations irrespective of differences in the GDP-binding capacity (see b). Lane M contains molecular mass markers. GDP-binding activity of extracts treated and assayed as reported in Methods (7500 c.p.m. correspond to 1 pmol of bound GDP). Note that in the pTu1 and pTu4 assays the maximum preincubation temperature was 95 °C instead of 90 °C. The results of four to six separate assays are shown.

were also prepared from cells containing the control plasmid pTu6. In preliminary assays, all extracts, except those derived from cells containing plasmids pTu5 and pTu6, were found to exhibit an appreciable GDP-binding capacity following incubation at 65 °C (data not shown). As the pTu5-encoded protein (Fig. 1) still contains the three consensus elements that are required to form the nucleotide-binding pocket, but lacks GDP-binding activity, the minimum EF-Tu sequence required for GTP/GDP binding is borne by plasmid pTu4. By comparison with the *E. coli* sequence (An & Friesen, 1980; Yokota *et al.*, 1980), the polypeptide encoded by the latter plasmid was found to correspond to the EF-Tu G domain (residues 1–200) (Parmeggiani *et al.*, 1987). Importantly, extracts from *E. coli* cells harbouring the pTu6 plasmid were completely devoid of GDP-binding activity at 65 °C, as expected if *E. coli* EF-Tu is completely denatured at this temperature.

In order to investigate the heat stability of the truncated EF-Tu proteins, aliquots of the S100 fraction (see Methods) were incubated at various temperatures be-

tween 65 °C (the temperature required for activation of overexpressed *T. maritima* EF-Tu) and 95 °C; the GDP-binding capacity of the preheated samples was then assayed at a constant temperature of 65 °C.

The gel electrophoretic patterns in Fig. 2(a) show that the products of the four recombinant plasmids are expressed to different degrees. Namely, the gel patterns of heat-treated S100 from *E. coli* cells harbouring either the pTu1 or the pTu4 plasmids display a prominent extra band that resists heating at 95 °C (pTu1) and 85 °C (pTu4). Although no extra bands are discernible in extracts prepared from cells harbouring either pTu2 or pTu3, the production of heterologous (*T. maritima*), thermally stable, protein by the two latter clones can still be inferred by the GDP-binding capacity of the extracts (Fig. 2b).

The results of GDP-binding assays carried out using several independent S100 preparations of each of the four recombinant clones are shown in Fig. 2(b). Variations in the GDP-binding capacity of the different preparations obtained from the same recombinant clone are probably accounted for by different activation levels attained by the

Fig. 3. Diagram of the genes encoding the parental and chimeric EF-Tu and G domain. Only the relevant restriction sites are indicated. Solid and dashed lines below the genes represent the encoded products.

recombinant proteins during preheating and, to a minor extent, by differences in protein content of the parent cell-free extracts ($8\text{--}12\ \mu\text{g protein }\mu\text{l}^{-1}$) (see Methods) or by different expression levels of the heterologous protein. Despite differences in the activity levels, however, independent preparations obtained from the same parent clone exhibit similar thermal-inactivation profiles. Indeed, the pTu1-encoded protein (full-length EF-Tu) is still apparent in the gel electrophoretic patterns of extracts heated up to $95\ ^\circ\text{C}$ (Fig. 2a) and its GDP-binding activity is not abolished following heating (Fig. 2b).

The plots in Fig. 2(b) also show that the plasmid pTu2-encoded protein (aa 1–316), which lacks the last 86 C-terminal residues of the EF-Tu appears to be less heat stable than the full-length protein: its activity is completely abolished by heating at $90\ ^\circ\text{C}$ and greatly reduced by heating at $85\ ^\circ\text{C}$ (as deduced by GDP-binding assays). No further reduction of the protein's stability occurs upon removal of 40 additional residues (plasmid pTu3, aa 1–276). Also, no further decrease in thermal stability is observed by extending deletions up to clone pTu4 which encodes the *T. maritima* G domain. In fact, the isolated G domain (aa 1–206) seems to be more heat stable than the pTu2- and pTu3-encoded proteins, although somewhat less stable than the full-length EF-Tu. This latter point is inferred by the fact that the protein is still abundant following heating of the extracts at $85\ ^\circ\text{C}$, but totally absent following heating at $95\ ^\circ\text{C}$.

The different stabilities of the three truncated proteins can be interpreted on the basis of the known three-dimensional structure of *E. coli* EF-Tu (Kjeldgaard & Nyborg, 1992). This appears to comprise three domains [aa 1–200 (G domain), aa 209–299 (domain II) and aa 300–393 (domain III)] whose interactions are important for the global function of the protein, and its overall structure is basically conserved in the thermophilic bacterium *Thermus*

thermophilus (Berchtold *et al.*, 1993). Although the native EF-Tu is required for tRNA binding to ribosomes, the three isolated domains do possess some of the EF-Tu activities. Namely, the *E. coli* G domain prepared using a genetically engineered *tufA* (Parmeggiani *et al.*, 1987) has been shown to retain certain properties of the intact molecule such as GDP and GTP-binding capacity and to display an intrinsic GTPase activity. Domain II/III (middle and C-terminal domains), isolated as a single polypeptide by tryptic digestion of the *Thermus thermophilus* EF-Tu, has been shown to maintain the ability to bind the elongation factor Ts and to be essentially refractory to proteolytic attack (Peter *et al.*, 1990). As the isolated domains, although stable, are more prone to thermal denaturation than the intact protein, it seems that the ability of the thermophile EF-Tu to withstand temperatures up to $95\ ^\circ\text{C}$ rests not only on specific amino acid preferences, but also on stabilizing co-operative interactions between the three domains. As the truncated *Thermotoga maritima* EF-Tus encoded by plasmids pTu2 and pTu3 lack either a segment of domain III (pTu2), or the entire domain III and part of domain II (pTu3), they are probably unable to attain the correct tertiary folding and, for this reason, they are more susceptible to thermal denaturation. In addition, as all the *tuf* genes possess an identical upstream region comprising promoter and ribosome-binding site, the low expression levels of the recombinant protein in cells harbouring plasmids pTu2 and pTu3 can be accounted for by degradation of the gene products by proteases of the mesophile host. If this is the case, the relative abundance of *T. maritima* G domain in *E. coli* cells harbouring the pTu4 plasmid could reflect a more compact tertiary packing, rendering the protein less susceptible to proteolytic attack.

As expected, no GDP-binding activity, and no extra bands, are discernible in S100 extracts prepared from *E. coli* cells containing the control plasmid pTu6 (Fig. 2a, b).

Fig. 4. SDS-PAGE of the parental (*T. maritima*) and chimeric (*T. maritima*/*E. coli*) proteins expressed in *E. coli*. Extracts from *E. coli* cells transformed with plasmids (a) pTu1; (b) plasmid harbouring the chimeric *tuf* gene; (c) pTu4; and (d) plasmid harbouring the chimeric G domain gene. Samples were treated as reported in Fig. 2. Arrows indicate the position of the expressed proteins. Lane M contains molecular mass standards.

Construction of chimeric *tuf* genes and analysis of the encoded products

Since a unique *Bcl*I site lies at an identical position (nucleotide 273) in both the *T. maritima* (Bachleitner *et al.*, 1989) and the *E. coli* (An & Friesen, 1980; Yokota *et al.*, 1980) *tuf* gene sequences, hybrid genes were constructed to assess the contribution of the first 90 amino acids to the heat stability of both the *T. maritima* EF-Tu and the isolated G domain. To this end, the recombinant plasmids pTu1, pTu4 and pTuB (harbouring the *E. coli tufB* gene) were digested with *Xba*I and *Bcl*I and the resultant fragments were ligated to generate the chimeras shown in Fig. 3.

SDS-PAGE analysis of crude extracts obtained from *E. coli* cells transformed with recombinant plasmids encoding a chimeric EF-Tu, or a chimeric G domain, displayed an extra band of molecular mass about 48 or 30 kDa, respectively (Fig. 4b, d). Incubation of the extracts at increasing temperatures (up to 85–90 °C) showed that replacement of the N-terminal portion (90 aa) of the thermophile proteins with the corresponding portion of their mesophile (*E. coli*) counterpart does affect the protein's thermal stability. As Fig. 4(a, b) shows, while the protein band (arrow in Fig. 4) corresponding to the *T. maritima* EF-Tu tolerates heating at 85 °C, the chimeric

protein begins to disappear following incubation of the extracts at 70 °C. Therefore, this region of the *T. maritima* EF-Tu harbours sequence element(s) which are essential for the maintenance of its thermally stable conformation. When the same analysis was extended to the G domain (Fig. 4c, d), which lacks the co-operative interactions with domains II and III, the effect of temperature on protein stability was more dramatic, the chimeric protein being as heat labile as the mesophile EF-Tu.

The authenticity of the chimeric EF-Tu was confirmed by testing the ability of cell-free extracts to bind GDP at 65 °C (data not shown). The same experiment was not feasible in the case of the chimeric G domain, as the protein was completely inactivated at 65 °C and a GDP-binding assay done below this temperature did not allow discrimination of the chimeric G domain and the host EF-Tu activities. Accordingly, separation of this chimeric protein from the *E. coli* EF-Tu was undertaken.

Extracts prepared from *E. coli* cells harbouring the recombinant plasmid encoding the chimeric G domain were chromatographed on a DEAE-cellulose column and the fractions were tested for GDP-binding activity at 30 °C. As Fig. 5 shows, two activity peaks were detected and identified as the *E. coli* EF-Tu and the chimeric G domain, respectively, on the basis of their SDS-PAGE

Fig. 5. DEAE-cellulose chromatography of extracts prepared from *E. coli* cells transformed with the plasmid bearing the chimeric G domain sequence. (a) GDP-binding activity of the eluted fractions assayed at 30 °C on 10 μ l aliquots; (b) SDS-PAGE analysis of 10 μ l aliquots of the same samples.

Fig. 6. Effect of preincubation at increasing temperatures on the GDP-binding capacity of the partially purified chimeric G domain. The samples were treated as described in Fig. 2 and the GDP-binding activity was assayed at 30 °C. NT, Not treated.

mobility. The partially purified chimeric G domain was then incubated at increasing temperatures (between 45 and 90 °C). As Fig. 6 shows, the GDP-binding activity of the chimeric protein was drastically reduced following incubation at 60 °C and disappeared altogether upon incubation at 65 °C in accordance with the gel electrophoretic patterns of crude extracts in Fig. 4(d).

On the whole, the results in Fig. 4 indicate that the thermal stability of both the thermophilic EF-Tu and the G domain are crucially dependent upon residues situated in the N-terminal region of the molecule, within the first half of the G domain. However, we have found that replacement of the first 90 residues of the mesophile (*E. coli*) EF-Tu with the corresponding region of the *T. maritima* protein does not result in increased heat stability (data not shown). We interpret this result as indicating that the remarkable heat resistance of the *T. maritima* EF-Tu is critically dependent upon unique tertiary structural interactions between certain N-terminal residues and distal residues that are absent in the *E. coli* moiety of the hybrid protein.

Conclusions

Primary structural comparison and engineering of the *tuf* gene sequence of the thermophile *T. maritima* allow the following conclusions. (1) Interactions between the three domains constituting the native EF-Tu play a role in maintaining the heat stability of the molecule. This is supported by the evidence that the isolated G domain of *T. maritima* EF-Tu is slightly less stable than the full-length protein, in accordance with the situation in *E. coli* (Jensen *et al.*, 1989). (2) Sequence element(s) that are crucial for heat stability appear to lie within the N-terminal portion of the *T. maritima* molecule between residues 1 and 90. This is borne out by the fact that replacement of the first 90 amino acids of both the isolated G domain and the full-length EF-Tu with the corresponding sequence of a mesophile (*E. coli*) EF-Tu gives rise to heat-labile proteins that do not tolerate heating at temperatures above 65 °C (EF-Tu) and 60 °C (G domain). (3) The N-terminal residues involved in thermal stability defy detection, as no amino acid preferences (or unique sequence motifs) appear to distinguish the *T. maritima* EF-Tu from the corresponding EF-Tu of mesophilic and other thermophilic (*Thermus thermophilus* and *Aquifex pyrophilus*) bacteria. Regardless of which residue(s) are involved, however, these appear to act through interaction with distal residues of the molecule. This interpretation is suggested by the fact that hybrid proteins comprising the first 90 residues of *T. maritima* EF-Tu and the remaining residues of *E. coli* EF-Tu are thermally labile, as would be expected if the *E. coli* moiety lacks putative residues involved in (stabilizing) interactions with residues situated in the *T. maritima* N-terminal portion of the molecule.

The data encourage random mutagenesis experiments focused on the initial portion of the *T. maritima* *tuf* gene. Combined functional and structural studies of the modified EF-Tus may cast light on the mechanism(s) that enable the thermophile factor to retain catalytic activity near the temperature of boiling water.

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REFERENCES

- Adams, M. W. W. (1993). Enzymes and proteins from organisms that grow near and above 100 °C. *Annu Rev Microbiol* **47**, 627–658.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- An, G. & Friesen, J. D. (1980). The nucleotide sequence of *tufB* and four nearby tRNA structural genes of *Escherichia coli*. *Gene* **12**, 33–39.
- Argos, P., Rossmann, M. G., Grau, U. M., Zuber, H., Frank, G. & Tratschin, J. D. (1979). Thermal stability and protein structure. *Biochemistry* **18**, 5698–5703.
- Bachleitner, M., Ludwig, W., Stetter, K. O. & Schleifer, K. H. (1989). Nucleotide sequence of the gene coding for the elongation factor Tu from the extremely thermophilic eubacterium *Thermotoga maritima*. *FEMS Microbiol Lett* **57**, 115–120.
- Berchtold, H., Reshetnikova, L., Reiser, C. O. A., Schirmer, N. K., Sprinzl, M. & Hilgenfeld, R. (1993). Crystal structure of active elongation factor Tu reveals major domain rearrangements. *Nature* **365**, 126–132.
- Bernardi, A. & Bernardi, F. (1979). Construction *in vitro* of hybrid plasmids carrying all the *EcoRI* fragments from *lrf^d18* DNA. *Eur J Biochem* **95**, 391–398.
- Bocchetta, M., Ceccarelli, E., Creti, R., Sanangelantoni, A. M., Tiboni, O. & Cammarano, P. (1995). Arrangement and nucleotide sequence of the gene (*fus*) encoding elongation factor G (EF-G) from the hyperthermophilic bacterium *Aquifex pyrophilus*: phylogenetic depth of hyperthermophilic bacteria inferred from analysis of the EF-G/*fus* sequences. *J Mol Evol* **41**, 803–812.
- Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heyneker, H. L., Boyer, H. W., Crosa, J. H. & Falkow, S. (1977). Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* **2**, 95–113.
- Burggraf, S., Olsen, G. J., Stetter, K. O. & Woese, C. R. (1992). A phylogenetic analysis of *Aquifex pyrophilus*. *Syst Appl Microbiol* **15**, 352–356.
- Buttarelli, F. R., Calogero, R. A., Tiboni, O., Gualerzi, C. O. & Pon, C. L. (1989). Characterization of the *str* operon genes from *Spirulina platensis* and their evolutionary relationship to those of other prokaryotes. *Mol Gen Genet* **217**, 97–104.
- Dagert, M. & Ehrlich, S. D. (1979). Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. *Gene* **6**, 23–28.
- Dever, T. E., Glynias, M. J. & Merrick, W. C. (1987). GTP-binding domain: three consensus sequence elements with distinct spacing. *Proc Natl Acad Sci USA* **84**, 1814–1818.
- Fabry, S., Lang, J., Niermann, T., Vingron, M. & Hensel, R. (1989). Nucleotide sequence of the glyceraldehyde-3-phosphate dehydrogenase gene from the mesophilic methanogenic archaeobacteria *Methanobacterium bryantii* and *Methanobacterium formicicum*. Comparison with the respective gene structure of the closely related extreme thermophile *Methanothermobacter fervidus*. *Eur J Biochem* **179**, 405–413.
- Higuchi, R., Stang, H. D., Browne, J. K., Martin, M. O., Huot, M., Lipeles, J. & Salser, W. (1981). Human ribosomal RNA gene spacer sequences are found interspersed elsewhere in the genome. *Gene* **15**, 177–186.
- Jensen, M., Cool, R. H., Mortensen, K. K., Clark, B. F. C. & Parmeggiani, A. (1989). Structure–function relationships of elongation factor Tu. Isolation and activity of the guanine-nucleotide-binding domain. *Eur J Biochem* **182**, 247–255.
- Kjeldgaard, M. & Nyborg, J. (1992). Refined structure of elongation factor EF-Tu from *Escherichia coli*. *J Mol Biol* **223**, 721–742.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Love, D. R. & Streiff, M. B. (1987). Molecular cloning of a β -glucosidase gene from an extremely thermophilic anaerobe in *E. coli* and *B. subtilis*. *Biotechnology* **5**, 384–387.
- Marck, C. (1988). 'DNA Strider': a 'C' program for the fast analysis of DNA and protein sequences on the Apple Macintosh family of computers. *Nucleic Acids Res* **16**, 1829–1836.
- Nosoh, Y. & Sekiguchi, T. (1990). Protein engineering for thermostability. *Trends Biotechnol* **8**, 16–20.
- Parmeggiani, A., Swart, G. W. M., Mortensen, K. K., Jensen, M., Clark, B. F. C., Dente, L. & Cortese, R. (1987). Properties of a genetically engineered G domain of elongation factor Tu. *Proc Natl Acad Sci USA* **84**, 3141–3145.
- Peter, M. E., Reiser, C. O. A., Schirmer, N. K., Kiefhaber, T., Ott, G., Grillenbeck, N. W. & Sprinzl, M. (1990). Interaction of the isolated domain II/III of *Thermus thermophilus* elongation factor Tu with the nucleotide exchange factor EF-Ts. *Nucleic Acids Res* **18**, 6889–6893.
- Russel, D. R. & Bennet, G. N. (1982). Construction and analysis of *in vivo* activity of *E. coli* promoter hybrids and promoter mutants that alter the –35 to –10 spacing. *Gene* **20**, 231–243.
- Sanangelantoni, A. M., Forlani, G., Ambroselli, F., Cammarano, P. & Tiboni, O. (1992). The *glnA* gene of the extremely thermophilic eubacterium *Thermotoga maritima*: cloning, primary structure, and expression in *Escherichia coli*. *J Gen Microbiol* **138**, 383–393.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.
- Schultes, V., Deutzmann, R. & Jaenicke, R. (1990). Complete amino-acid sequence of glyceraldehyde-3-phosphate dehydrogenase from the hyperthermophilic eubacterium *Thermotoga maritima*. *Eur J Biochem* **192**, 25–31.
- Seidler, L., Peter, M., Meissner, F. & Sprinzl, M. (1987). Sequence and identification of the nucleotide binding site for the elongation factor Tu from *Thermus thermophilus* HB8. *Nucleic Acids Res* **15**, 9263–9277.
- Tiboni, O., Di Pasquale, G. & Ciferri, O. (1978). Purification of the elongation factors present in spinach chloroplasts. *Eur J Biochem* **92**, 471–477.
- Tiboni, O., Sanangelantoni, A. M., Cammarano, P., Cimino, L., Di Pasquale, G. & Sora, S. (1989). Expression in *Escherichia coli* of the *tuf* gene from the extremely thermophilic eubacterium *Thermotoga maritima*: purification of the *Thermotoga* elongation factor Tu by thermal denaturation of the mesophile host-cell proteins. *System Appl Microbiol* **12**, 127–133.
- Vieira, J. & Messing, J. (1982). The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19**, 259–268.
- Weijland, A., Harmark, K., Cool, R. H., Anborgh, P. H. & Parmeggiani, A. (1992). Elongation factor Tu: a molecular switch in protein biosynthesis. *Mol Microbiol* **6**, 683–688.
- Yokota, T., Sugisaki, H., Takanami, M. & Kaziro, Y. (1980). The nucleotide sequence of the cloned *tufA* gene of *Escherichia coli*. *Gene* **12**, 25–31.

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