

Isolation and Characterization of a New Ribosomal Protein from the Thermophilic Eubacteria, *Thermus thermophilus*, *T. aquaticus* and *T. flavus*

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Summary: A ribosomal protein, showing no homology with other known prokaryotic ribosomal proteins, was isolated and characterized from the thermophilic eubacteria, *Thermus thermophilus*, *T. aquaticus* and *T. flavus*. This small (26 amino acids) and

strongly basic (1 acidic and 13 basic residues) protein displayed the same primary structure from all three sources. Interestingly, it shows about 65% homology with a ribosomal protein from spinach chloroplasts (J. Schmidt, personal communication).

Key terms: Thermophilic eubacteria, ribosomes, protein sequencing.

Ribosomes, present in all living cells, are responsible for translating the genetic code into polypeptide chains. Although these organelles have been thoroughly characterized by a variety of chemical, physical, immunological and genetic techniques, elucidation of the molecular mechanism of protein biosynthesis is still hampered by the lack of a molecular model. Diffracting crystals have been prepared from the ribosomes of thermophilic bacteria; such ribosomes are very stable and therefore easier to crystallize than those from other sources.

Nevertheless, little is known about the primary sequences of thermophilic ribosomal proteins, and such information is essential for the construction of a molecular model. The best thermophilic bacterial ribosome crystals are obtained from the Gram-negative *Thermus thermophilus*. This organism was isolated in 1974^[1] and the high stability of its ribosomes is presumably linked to its optimal growth temperature of 75°C.

In the present work we report the complete amino-acid sequence of a very basic, low molecular mass ribosomal protein, which has the same primary sequence in *T. thermophilus*, *T. flavus* and *T. aquaticus*, and which shows no homology with any known ribosomal protein from *Escherichia coli*.

Materials and Methods

Reagents

Acetonitrile for HPLC was Lichrosolve grade from E. Merck (W-6100 Darmstadt). Polyvinylidene difluoride (PVDF) membranes were from Millipore (W-6078 Neu-Isenburg), acrylamide and urea from Bethesda Research Laboratories, USA. All other chemicals were supplied by E. Merck. Vydac column packing C4 (300 Å, 5 µm) was obtained from the separation group (Hesperia, CA, USA). Columns were packed at the Max-Planck-Institute for Molecular Genetics, Berlin.

Extraction of ribosomal proteins

70S ribosomes were isolated and ribosomal subunits were prepared as described in ref.^[2,3]. The total 30S subunit protein (TP-30) from *T. thermophilus* HB8 was extracted with either acetic acid^[4] or LiCl-urea^[5], dialysed extensively against 2% acetic acid containing 10mM 2-mercaptoethanol, then lyophilized. TP-30 proteins from *T. aquaticus* EP002276 (Porton Limited CAMR, Porton Down, Salisbury, U.K.) and *T. flavus* AT-62 were extracted with acetic acid as described in ref.^[4]. The proteins from all three sources were separated by reversed phase HPLC on a Vydac C4 (300 Å, 5 µm) column, using an acetonitrile gradient in aqueous 0.1% trifluoroacetic acid. Peak fractions were pooled and analysed on micro 2-dimensional gels as described in ref.^[5,7].

Identification of ribosomal proteins

The TP-30 proteins were identified by micro 2-dimensional polyacrylamide gel electrophoresis as described in ref.^[6,7], but with shorter electrophoresis times in both the first and second dimensions. The proteins are numbered according to ref.^[8], and in some cases according to their primary structural homology with known ribosomal proteins.

Abbreviations:

PVDF: Polyvinylidene difluoride; TP-30: total-protein from 30S; PTH: phenylthiohydantoin; TFA: trifluoroacetic acid.

Sequence determination

Sequences were determined in an Applied Biosystems pulsed liquid-phase sequencer, model 477A, equipped with a model 120 PTH-aa analyser. Samples were dissolved in 100% trifluoroacetic acid and applied as described previously^[9]. Sequences were also determined in the Knauer sequencer, model 810, by covalent attachment to PVDF membranes by the wet-phase degradation technique described in ref.^[10].

Mass spectrometry

The mass of the protein was determined in a triple quadrupole instrument from Finnigan, USA, model TSQ, equipped with electrospray ionization. Samples were dissolved in 10–20 μ l of 1% acetic acid in 50% (by vol.) methanol/water and injected directly into the capillary of the analyser. Solutions were vaporized inside the ionization cell under normal pressure, high voltage (4250 V) and a flow rate of 1 μ l/min.

Results and Discussion

Ribosome isolation

Ribosomes prepared by the method of Ohno-Iwashita et al.^[3] contain considerable quantities of cell membrane fragments, which prevents their use for structural analysis or protein identification. We therefore followed the method described in ref.^[2], which produces ribosomes without membrane contaminants.

Extraction and purification of ribosomal proteins

As described in Materials and Methods, the ribosomal proteins were extracted with acetic acid

and separated by reversed phase HPLC. Since several ribosomal proteins are known to lose C-terminal peptides by treatment with acetic acid (e.g. L6 from *E. coli*: T. Choli, Doctoral Thesis, Aristotle University of Thessaloniki, 1985), the proteins were also extracted with LiCl-urea as a control. The similar relative molecular masses (6000–30 000) and isoelectric points (8–10) of most of the ribosomal proteins make them difficult to separate^[11]. Classical purification methods for ribosomal proteins include combinations of ion exchange and ion exclusion chromatography. Protein yields from these laborious procedures are about 5% for the less easily isolated smaller proteins, up to 50% for the larger proteins^[12–15]. Reversed phase HPLC is, however, suitable for separating ribosomal proteins, because retention depends on hydrophobicity, rather than size or net charge. Reversed phase HPLC also has advantages of speed, high recovery and high sensitivity. Figs. 1, 2 and 3 show separations of TP-30 ribosomal proteins from *T. thermophilus*, *T. aquaticus* and *T. flavus*, respectively, using a Vydac C4 (300 Å, 10 μ m) with an acetonitrile-TFA gradient. The first small peak at about 20% acetonitrile is common to all three organisms, and represents the protein under investigation. Its early elution is due to its low molecular mass and extremely basic character.

In 2-dimensional gels it migrates very quickly, in accordance with its size and net charge (see protein identification, below).

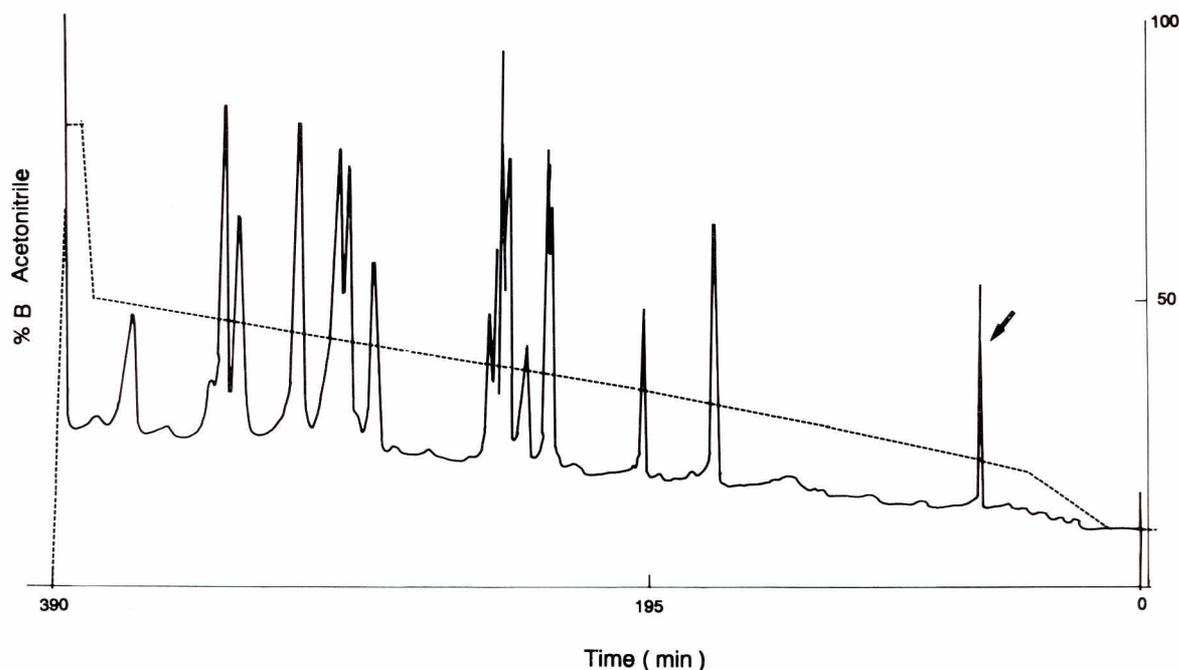


Fig. 1. RP-HPLC of TP-30 from *T. thermophilus*.

TP-30 (300 μ g) was dissolved in 1000 μ l 2% acetic acid, injected onto a Vydac C4 column (250 \times 4 mm) and chromatographed in a solvent system of 0.1% TFA/acetonitrile. The gradient applied was as indicated in the figure. Measurements were made at 220 nm, 0.16 arbitrary units (full scale), at a flow rate of 0.7 ml/min. The arrow corresponds to protein **Lix** ←

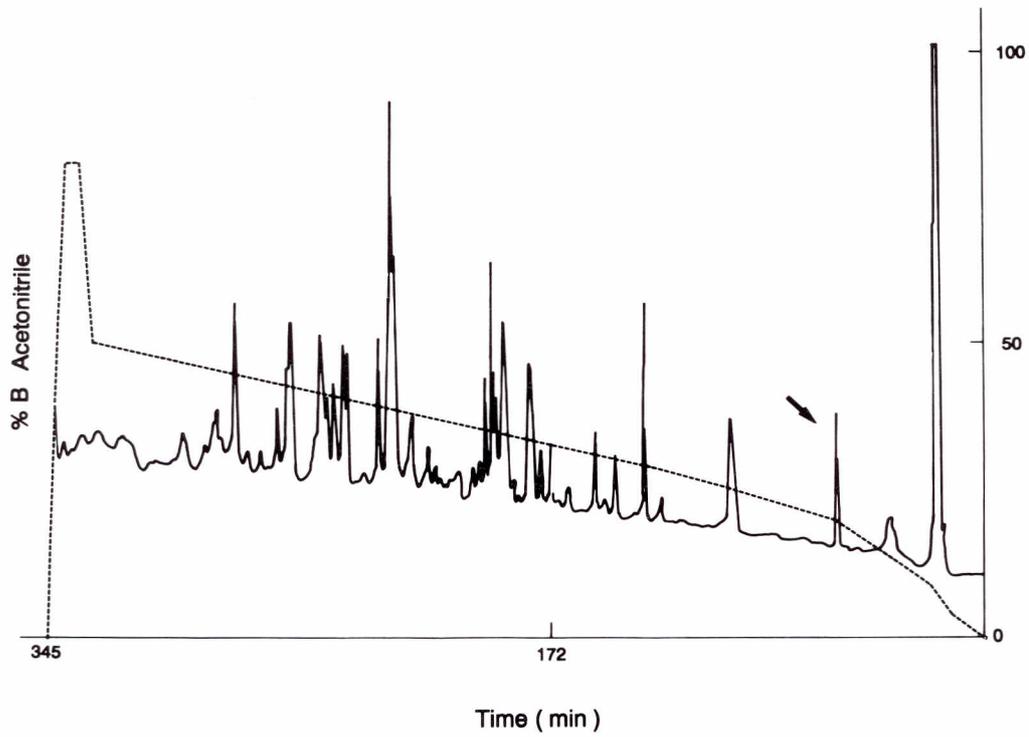


Fig. 2. RP-HPLC from *T. aquaticus*.
All conditions as in Fig. 1.

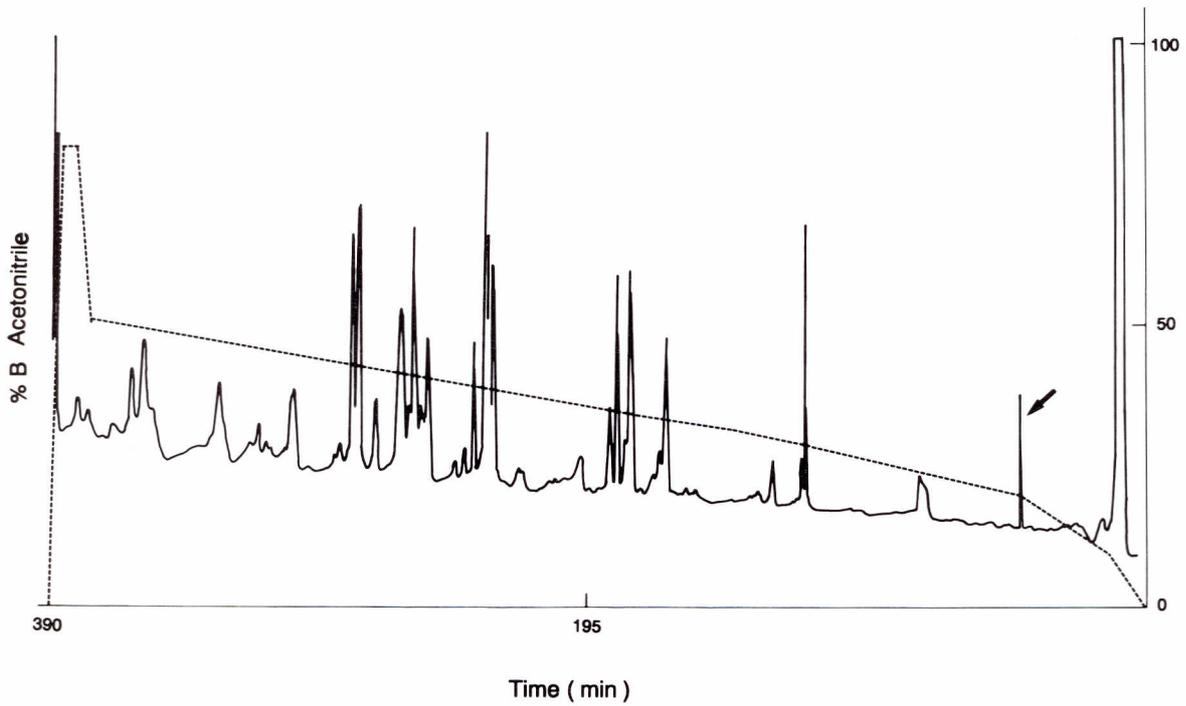


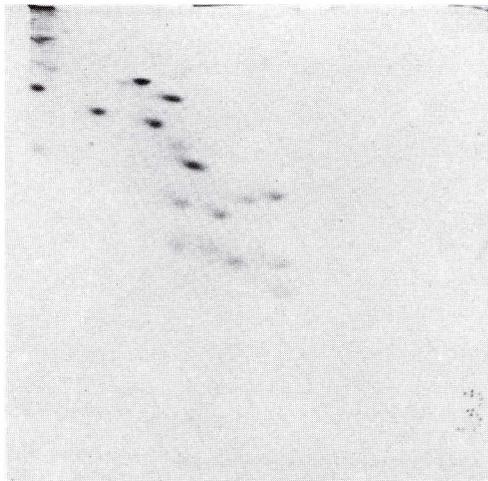
Fig. 3. RP-HPLC of TP-30 from *T. flavus*.
All conditions as in Fig. 1.

Protein identification

Recently Wada^[15,16] reported that 2-dimensional gel electrophoresis of *E. coli* ribosomal proteins in the Kaltschmidt-Wittmann system reveals 4 additional spots (2 distinct and 2 faint), if the gel is pre-run with a chemical scavenger to remove free radicals generated by the peroxide catalyst. Proteins of the two major spots (A and B) have been isolated and partly sequenced. We also used the electrophoresis system of Kaltschmidt-Wittmann, but with a shorter electrophoresis time than proposed in ref.^[6,7] in both dimensions. The procedure therefore produced an ineffi-

cient separation of most of the TP-30 ribosomal proteins, but it permitted the detection of a new small and very basic protein which migrated close to the dye front. It should also be pointed out that the urea and acrylamide were of electrophoresis grade purity in order to minimize the appearance of artifacts. Fig. 4A shows the electrophoretic patterns of TP-30 ribosomal proteins from *T. flavus* (left) and *T. aquaticus* (right), using the electrophoresis times suggested in ref.^[6,7]. As expected, the small basic protein runs out of the gels. Fig. 4B shows separations of the same proteins, using shorter electrophoresis

TP-30 Ribosomal proteins from *Thermus flavus*



Long run

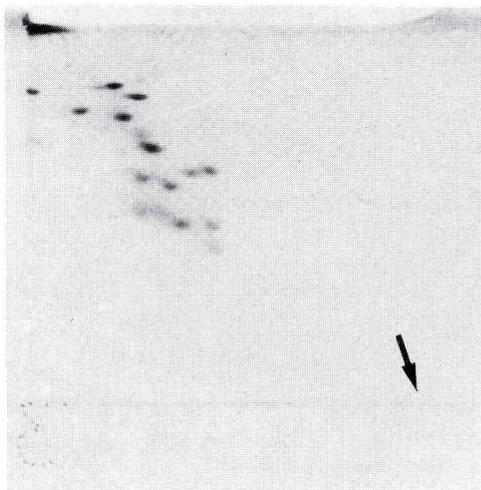
TP-30 Ribosomal proteins from *Thermus aquaticus*



Long run

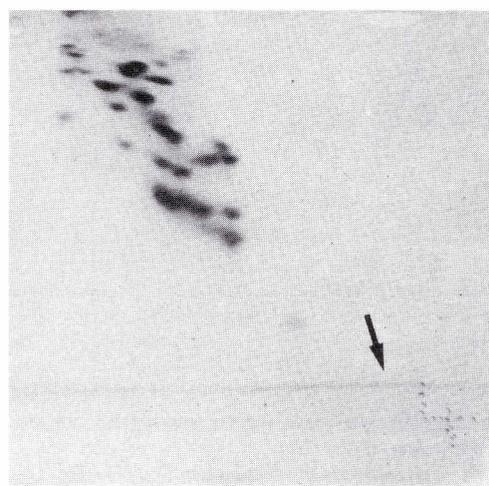
A

TP-30 Ribosomal proteins from *Thermus flavus*



Short run

TP-30 Ribosomal proteins from *Thermus aquaticus*

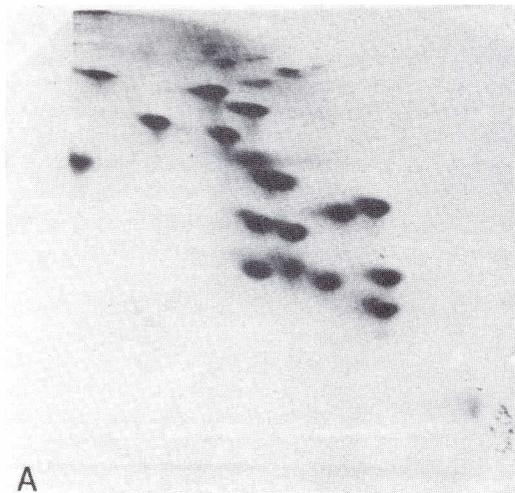


Short run

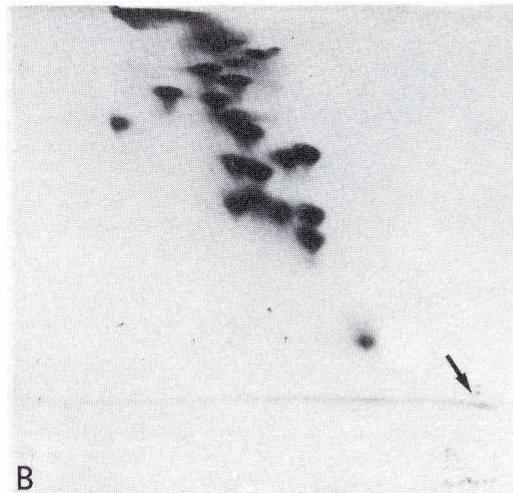
B

Fig. 4. Two-dimensional gel electrophoresis of TP-30 ribosomal proteins from *T. flavus* and *T. aquaticus* according to ref.^[6,7].

A) long electrophoresis time; B) short electrophoresis time. Proteins were extracted by the acetic acid method^[4]. This is marked by an arrow

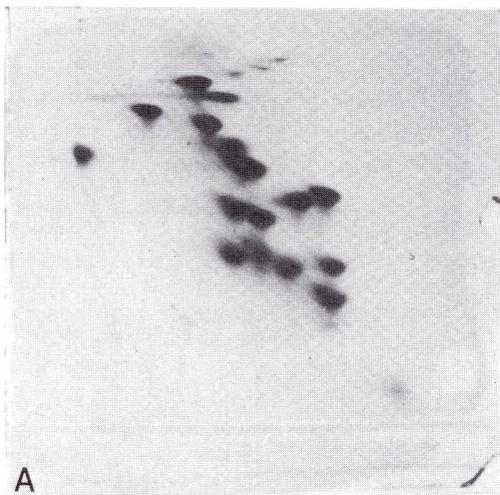
TP-30 Ribosomal proteins from *Thermus thermophilus*

Acetic acid extraction, long run

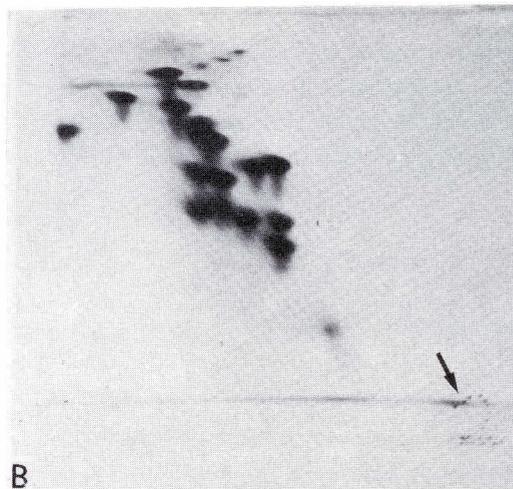
TP-30 Ribosomal proteins from *Thermus thermophilus*

Acetic acid extraction, short run

Fig. 5. Two-dimensional gels of TP-30 ribosomal proteins from *T. thermophilus* extracted by the acetic acid method. When a long electrophoresis time is used, the small basic protein runs out of the gels (A). With a short electrophoresis time protein Thx becomes visible (B). The arrow shows the spot corresponding to protein Thx.

TP-30 Ribosomal proteins from *Thermus thermophilus*

LiCl-urea extraction, long run

TP-30 Ribosomal proteins from *Thermus thermophilus*

LiCl-urea extraction, short run

Fig. 6. Two-dimensional gels of TP-30 ribosomal proteins from *T. thermophilus* extracted by the LiCl-urea method. All other conditions are as in Fig. 5. The arrow corresponds to protein Thx.

times. In both cases the small protein appears as a faint spot on the right hand side of the gels, very close to the dye front. Figs. 5A and 6A show the ribosomal proteins extracted from the 30S subunits of *T. thermophilus* with acetic acid^[4] and with LiCl-urea^[5], respectively. In both cases the number of extracted proteins is identical, showing that the acidic conditions did not

lead to protein fragmentation. On the other hand, the electrophoresis time is longer, so that the small protein is not detected. In Figs. 5B and 6B the electrophoresis time was shorter: in both cases the small protein is present, thus providing strong evidence that it is not an artifactual fragment produced by the acidic extraction conditions.

TP-30 Ribosomal proteins from *Thermus thermophilus*

Electrotransfer of TP-30 ribosomal proteins onto PVDF membranes, short run

Fig. 7. Electrotransfer of TP-30 ribosomal proteins from *T. thermophilus* onto PVDF membranes according to refs.^[18,19].

The two-dimensional electrophoresis was performed under the same conditions as in Figs. 5B and 6B. The arrow shows the faint comassie blue-stained spot which corresponds to protein Thx.

Sequence determination

The primary structure was elucidated as described in Materials and Methods. The same primary structure was found for the protein blotted onto PVDF membranes^[17,18] (Fig. 7) and the protein eluted from re-

versed phase HPLC (Figs. 1, 2 and 3), indicating that the faint spot in the electropherogram is not an artifact. Fig. 8 shows the sequence of the small protein from *T. thermophilus*, *T. flavus* and *T. aquaticus*. It contains 26 amino-acid residues (13 arginines plus

<i>T. thermophilus</i>	G	K	G	D	⁵ R	R	T	R	R	¹⁰ G	K	I	W	R	¹⁵ G	T	Y	G	K	²⁰ Y	R	P	R	K	²⁵ K	K
<i>T. flavus</i>	G	K	G	D	R	R	T	R	R	G	K	I	W	R	G	T	Y	G	K	Y	R	P	R	K	K	K
<i>T. aquaticus</i>	G	K	G	D	R	R	T	R	R	G	K	I	W	R	G	T	Y	G	K	Y	R	P	R	K	K	K

Fig. 8. Amino-acid sequence of Thx proteins from *T. thermophilus*, *T. aquaticus* and *T. flavus*.

The sequences were derived by automatic sequencing using a pulsed-gas-liquid phase ABS-sequencer or a Knauer sequencer after covalent attachment to PVDF membranes according to ref.^[10].

<i>T. thermophilus</i>	G	K	G	D	⁵ R	R	T	R	R	¹⁰ G	K	I	W	R	¹⁵ G	T	Y	G	K	²⁰ Y	R	P	R	K	²⁵ K	K
		:				:		:		:	:	:	:	:	:		:		:	:		:	:		:	
Chloroplasts	G	R	G	D	R	K	T	A	K	G	K	R	F	N	H	S	F	G	N	A	R	P	K	N	K	N
Chloroplasts	K	G	R	³⁰ G	P	?	P	K	³⁵ A	?	A	I	F	⁴⁰ F	K	G	D									

Fig. 9. Structural homology between Thx and a recently isolated protein from spinach chloroplasts using the GCG-Programm "Bestfit".

The computer analysis was based on the GCG software (version 7.2) sequence comparison using NBRF/Swiss Prot or Ribo (MPI) databanks with a VAX 8600/VMS computer.

lysines, and a single acidic residue of aspartic acid). Its isoelectric point of 12.1 is in agreement with its position in 2-dimensional electropherograms. The amino-acid sequence as determined by Edman chemistry gives a calculated relative molecular mass of 3205.9 ± 0.5 . This agrees closely with the value of 3205.8 determined by mass spectrometry, using an electrospray quadrupole instrument. We could find no homology with any other known prokaryotic protein. The new protein was therefore named Thx. Interestingly, Thx possesses about 65% homology and 45% identity with a larger protein isolated recently from the 70S ribosomes of spinach chloroplasts (J. Schmith, personal communication; sequence shown in Fig. 9). The fact that the protein has been isolated both from eubacterial thermophilic and chloroplast ribosomes is a strong evidence that it is not an impurity but a new ribosomal protein, which shows no homology with any known ribosomal protein. Further studies of the function of these proteins are necessary to determine the relevance of their structural relatedness.

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