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Stabilisation of some of the protein synthesis components in the thermophilic fungus, *Humicola lanuginosa*

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Abstract The thermal stabilities of tRNA from the thermophilic fungus, *Humicola lanuginose* were compared with that from the mesophilic yeast, *Candida utilis,* by measuring the increase in the optical density with temperature. tRNAs from both the species were stable in the presence of millimolar quantities of magnesium chloride upto 50°C, the optimum growth temperature of the fungus. Aminoacyl tRNA synthetases were maximally active at 40°C under the *in vitro* assay conditions. They were fractionated and one species of valine tRNA synthetase was purified to homogeneity. The purified enzyme was protected against inactivation to varying degrees when preincubated with the substrates valine, tRNA and ATP as well as spermine. Protein turnover studies showed that the rate of turnover was higher at higher temperatures. It was concluded from these results that the protein synthesizing machinery of this fungus has no intrinsic stability but it is stabilised by intracellular factors. Higher rate of protein turnover also plays a role for growth at higher temperature.

Keywords. Thermophilic fungus; stabilisation of synthetase; rate of protein turnover; *Humicola lanuginosa.*

Introduction

A variety of microorganisms have the ability to grow optimally at temperatures much higher than the ordinary and some of them can grow well at the boiling point of water. But no eukaryote has been reported to grow at temperatures beyond 60°C. As both prokaryotes and eukaryotes comprise of aerobic organisms with simple nutritional requirements the inability of eukaryotes, especially fungi, to grow at temperatures beyond 60°C is surprising. Attempts have been made to understand the molecular basis of the upper temperature limit for the growth of eukaryotes. Tansey and Brock (1978) have suggested that thermophilic eukaryotes are unable to synthesise intracellular membranes that are functional beyond 60°C. However, experimental proof for such a postulate is lacking. Studies on the lipids of thermophilic species have shown that these species produce lipids with a high content of saturated fatty acids (Sumner and Morgan, 1969; Sumner *et al.,* 1969). These results suggest that the types of lipids the organism is able to produce determine the temperature of its growth.

The biological activity of proteins is of prime importance for growth at the optimum temperature of an organism. A large number of proteins from thermophilic bacteria, especially *Bacillus stearothermophilus* and *Thermus aquaticus* have been purified and characterised (Ljungdahl and Sherod, 1976; Amelunxen and Murdock, 1978). Some of these proteins have been found to be very stable while others not so stable. They have been classified into 4 groups on the basis of their thermostability *in vitro*. The proteins which are labile are postulated to be either stabilised by the

Abbreviation used: TCA, Trichloroacetic acid.

intracellular environment or replenished by synthesis during growth (Singleton and Amelunxen, 1973). The majority of proteins from thermophilic bacteria, however, are intrinsically stabler than the corresponding proteins from mesophilic organisms. Only a small number of proteins from thermophilic fungi have been studied. There is, in general, a lack of information on thermophilic eukaryotes and fungi, in particular. The available data, however, show that most intracellular proteins from thermophilic fungi, unlike that from thermophilic bacteria, are thermolabile *in vitro* around the growth temperature of the organism (Prasad and Maheshwari, 1978a; Wali *et al*., 1979). In contrast, many extracellular proteins from these organisms are thermostable (Loginova and Tashpulatov, 1967; Rao *et al.,* 1979, 1981). From these studies it appears that a large proportion of proteins from thermophilic fungi do not exhibit the thermostability required to grow at their optimum growth temperatures. It is interesting to know how these organisms carry out protein synthesis at their optimum temperatures of growth. The thermal characteristics of the protein synthetic machinery are of interest in this connection. We present here the results of our studies on the thermostability of tRNA, and aminoacyl-tRNA synthetases as well as protein turnover in *Humicola lanuginosa,* a commonly found thermophilic fungus which has an optimum temperature of growth at 50°C and upper temperature for growth at 60°C.

Materials and methods

Humicola lanuginosa was kindly provided by Dr. R. Maheshwari of this Department and the mesophilic yeast *Candida tropical* was from the Microbiology and Cell Biology Laboratory of this Institute. [^{14}C]-Algal protein hydrolysate (24 mCi/matom) and [^{14}C]-valine (30 mCi/mmol) were from Bhabha Atomic Research Centre, Bombay. [^{14}C]-L-Leucine (339 mCi/mmol) and [^{3}H]-L-leucine (18 Ci/mmol) were from Radiochemical Centre, Amersham, England. The ion-exchange materials and other fine chemicals were from Sigma Chemical Co., St. Louis, Missouri, USA. All other chemicals were of analytical grade.

Isolation of tRNA

H. lanuginosa cells were grown in a synthetic medium (Prasad and Maheshwari, 1978b) with L-asparagine concentration raised to 0·4% in a temperature controlled rotary shaker at 50°C. After 10-12 h the cells were harvested by filtering and tRNA was isolated by extraction with phenol (Von Ehrenstein, 1967). Contaminating polysaccharides were removed by selective precipitation with cetyltrimethylammoniumbromide (Bellamy and Ralph, 1968) and the polysaccharide-free tRNA was deacylated according to the method of Harris (1978). *C. utilis* was grown in an enriched medium at 30°C, the cells were collected by centrifugation and the tRNA was isolated in a similar manner.

Thermostability measurements

Thermal denaturation of tRNA was monitored by measuring the hyperchromicity at 260 nm with increase in temperature in 10 mM cacodylate buffer, pH 7·2 containing

5 mM NaCl and specified amounts of $MgCl_2$ or spermine. About one absorbance (260 nm) unit/ml of tRNA preparation in a final volume of 250 μ1 was used to monitor the increase in absorbance at 2°C increments in a Beckman DU-8B recording spectrophotometer.

Pancreatic RNase digestion

Susceptibility of tRNA to pancreatic RNase was monitored by measuring the increase in absorbance at 260 nm with time as indicated above. A known quantity of RNase A was mixed with 500 μ1 of the tRNA preparation (about one absorbance (260 nm) unit/ml) in 10 mM cacodylate buffer, pH 7·2 containing 60 mM KCl and 10 mM $MgCl_2$ and the increase in absorbance (260 nm) was monitored at a specified temperature as a function of time at one minute intervals.

Rate of protein turnover

The method used to study the rate of protein turnover was an adaptation of the double isotope method of Arias *et al.* (1969). A 20 ml medium was inoculated with freshly grown *H. lanuginosa* cells and grown for 6 h at 50°C in a rotary shaker. The cells were shifted to the specified temperature and after 5 min 5 μCi of [^{14}C]-L-leucine was added. After labelling for 30 min the cells were harvested by mild suction through millipore membrane and washed well with the growth medium. The cells were then suspended in 20 ml of fresh medium and allowed to grow for 2 h at the same temperature. This was to allow turnover of the [^{14}C] -label already incorporated into the protein. The cells were collected and an amount equal to that at the end of the [^{14}C] -pulse period was grown for 10 min with 25 μCi of [^{3}H] -L-leucine. The remaining cells were allowed to grow in the non-radioactive medium at the same temperature for 10 min. The two types of cells were mixed, filtered and washed thoroughly with ice-cold medium. The washed cells were ground with sand and the lysate was extracted with 5 ml of a buffer containing 50 mM Tris-HCl, pH 7·2 and 25 mM NaCl. The extract was centrifuged at 8,000 *g* for 10 min and an aliquot of the supernatant fraction was spotted on Whatman 3 mm paper and thoroughly washed with 10% hot trichloroacetic acid (TCA) containing 0·1% L-leucine and ethanol-ether mixture. The paper was dried and counted for [^{14}C]-and [^{3}H]-labels in a liquid scintillation counter. The less the number of [^{14}C] -counts at the end, the more will be the protein turnover. Thus the ratio [^{3}H]/[^{14}C]-is a measure of the rate of protein turnover.

Aminoacyl tRNA synthetase assay

H. lanuginosa cells (2-5 g), grown for 8-10 h were ground with an equal amount of sand and extracted with chilled buffer containing 10 mM Tris-HCl, pH 7·2, 10 mM $MgCl_2$, 30 mM NH_4C1, 5 mM β-mercaptoethanol and 10% glycerol. The cell lysate was centrifuged at low speed and the supernatant fraction was further centrifuged at 105,000 *g* for 1 h. The supernatant fraction thus obtained was freed of endogenous tRNA and amino acids by absorption to a DEAE-Sephacel column followed by

elution from the column with the extraction buffer containing 0·3 M KCl (Muench and Berg, 1966).

The standard assay mixture contained 50 mM Tris-HCl, pH 7·2, 2 mM ATP, 0·5 mM CTP, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0·5 mM EDTA, 0·1 μCi [^{14}C]-algal protein hydrolysate (24 mCi/m atom) about 4 absorbance (260 nm) units of tRNA and about 10μg of enzyme protein in a total volume of 200 μl. After incubation at 37°C for a specified period 150 μ1 of the reaction mixture was applied to a Whatman 3 mm paper, presoaked in 10% TCA and was washed thoroughly with TCA solution containing 0·1% casamino acids. The paper was dried and counted.

Valine tRNA synthetase assay was carried out in a similar manner except that the composition of the assay mixture was different. It contained 50 mM Tris-HCl, pH 8·0, 10 mM KCl, 3 mM ATP, 15 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0·5 mM EDTA, 180 μM [^{14}C]-L-valine (30 mCi/mmol) about 50 absorbance (260 nm) units of tRNA and about 170 ug of purified valine tRNA synthetase in a final volume of 200 μ1.

Results

The stability of tRNA

The stability of the tRNAs is an important factor which determines the upper temperature of growth of an organism. Hence the thermal melting profiles of total tRNA from *H. lanuginosa* were compared with that of mesophilic yeast, *C. utilis*. Increase in the optical density was measured against temperature in the buffer with different concentrations of $MgCl_2$ or spermine. tRNA from both sources had almost identical melting profiles suggesting similar stability. Although *H. lanuginose* tRNA melted at temperatures lower than the optimum temperature of its growth in the absence of any added $MgCl_2$ or spermine, addition of small quantities of these raised the temperature of melting. In 2·5 mM $MgCl_2$ the temperature at which *H. lanuginosa* tRNA started melting was 56°C and the midpoint of melting was 76°C. The tRNA was thus stable at the optimum temperature of its growth, but did not have any special feature different from that of *C. utilis* tRNA which was also stable up to about 56°C (figure 1A).

Rate of digestion of a nucleic acid with a nuclease roughly indicates the degree of compactness of its structure. The tRNA samples were digested with RNase A at different temperatures to get an insight into their tertiary structure. *H. lanuginose* tRNA was digested at a slower rate by the enzyme (figure 1B).

Stability of aminoacyl tRNA synthetases

Studies were carried out with the total synthetase preparation, freed of the endogenous tRNA and amino acids by batch-wise elution from DEAE-Sephacel, to establish the optimum conditions for assay. Thus the pH, the concentration of ATP, magnesium ions and other components as well as the time of incubation for linear response were determined using ^{14}C-algal protein hydrolysate (data not presented). The effect of temperature on the aminoacylation reaction was studied by carrying out the reaction at various temperatures. The acceptance increased with temperature

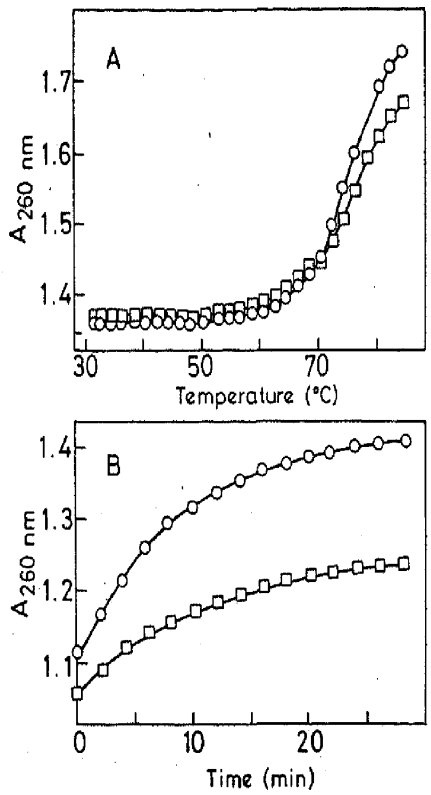

Figure 1. Melting profile of tRNA and the rate of digestion with RNase. **A.** Melting profile of tRNA: a solution of tRNA in 10 mM bufer, pH 7·2, containing 5 mM NaCl and 2·5 mM $MgCl_2$ was taken in a 250 μl cuvette and absorbance against temperature was recorded in a DU-8B Beckman Recording Spectrophotometer at 2 min intervals. **B.** Rate of digestion with pancretic R Nase: 2·5 μg of RNase was.mixed with 500 μl of tRNA preparation in 10 mM cacodylate buffer, pH 7·2, containing 60 mM KCl and 10 mM $MgCl_2$ at 37°C. Increase in absorbance was monitored as a function of time at 1 min intervals. (□), *H, lanuginosa* tRNA; (O), *C. untilis* tRNA.

from 30°C to 40°C and then decreased sharply with rise in temperature (figure 2). The activity at 50°C, the temperature for optimum growth of the organism, was only about half of that at 40°C and at 60°C, the maximum growth temperature was very low. These results suggested that in the *in vitro* reaction all the factors that stabilize the enzymes were not present. It was also not clear whether the *in vitro* lower activity at higher temperatures was due to reversible thermal inactivation or due to irreversible thermal denaturation of the enzymes. Only by the use of a homogeneous single enzyme this could be clarified. The synthetase preparation was therefore fractionated by ammonium sulphate precipitation, chromatography on CM-50 Sephadex (von der Haar, 1979) interfacial salting out on a Sepharose-4B column by a reverse gradient (von der Haar, 1976) and affinity chromatography. Upon chromatography on the CM-50 Sephadex column valine tRNA synthetase resolved into two peaks, a minor peak (valine tRNA $synthetase_1$) and a major peak (valine tRNA $synthetase_2$). On further fractionation valine tRNA $synthetase_2$ gave a single protein as shown by electrophoresis on Polyacrylamide gels under native and denaturing conditions. This purified species was used for further studies. Valine tRNA $synthetase_1$ could not,

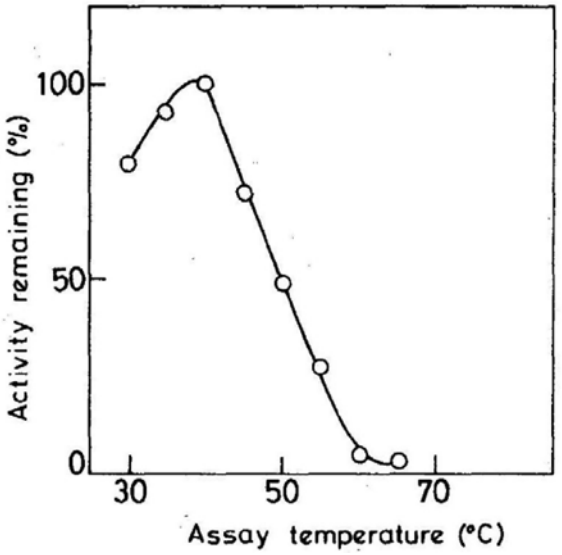

Figure 2. Effect of temperature on aminoacyl tRNA synthetase activity.
The aminoacylation assay for total synthetase was carried out at different temperatures using [^{14}C]-algal protein hydrolysate. The maximum activity obtained was taken as 100% to plot the values at different temperatures.

however, be purified to a homogeneous state by this procedure. Details of the purification of valine tRNA synthetase2 will be published elsewhere.

Studies with valine tRNA synthetase$_2$

Experiments were again carried out to establish the optimum conditions for assay with total tRNA using [^{14}C]-L-valine for label (data not presented). Study of the effect of temperature on the aminoacylation reaction showed that as in the case of the total tRNA synthetase preparation the maximum activity of valine tRNA synthetase$_2$ was below the optimum growth temperature of the organism (see figure 2). However, the maximum activity was at 45°C, slightly higher than that for the total tRNA synthetase. At 50°C the activity was nearly 85% of that at 45°C but at 60°C the activity was only about 10% of that at 45°C. In order to distinguish whether the low activity at higher temperatures was due to heat inactivation or irreversible thermal denaturation of the protein, the following experiments were carried out. The enzyme was heated at different temperatures in the presence of various effector agents for 5 min, cooled and assayed at 40°C. In the absence of the substrates at the time of preincubation the enzyme was highly thermolabile. It lost more than 50% activity by heating for 5 min at 40°C and more than 90% by heating at 50°C. It was clear that the enzyme was irreversibly inactivated. The substrates, L-valine, tRNA and ATP were able to protect the enzyme to some extent against heat denaturation individually and in combination. Among the substrates tRNA was the most effective. These substrates in combination could give a great deal of protection to the enzyme (figure 3). In addition, spermine in very small quantities could also protect the enzyme to some extent (data not presented).

Heat denaturation experiment was carried out by an alternate method to get an

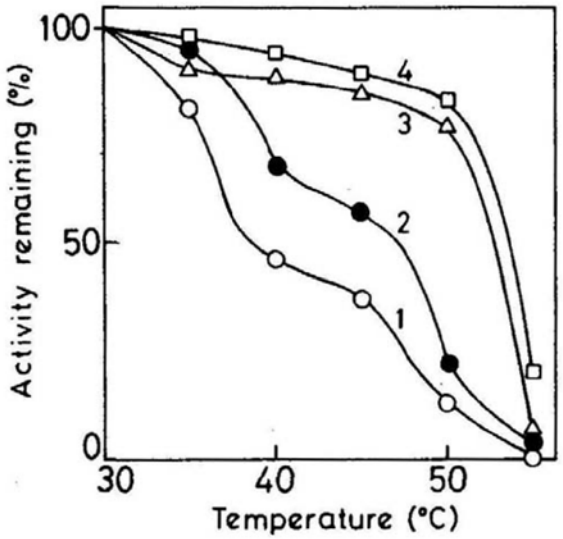

Figure 3. Protection of valine tRNA synthetase$_2$ by substrates at different temperatures.
Valine tRNA synthetase$_2$ in 50 mM Tris-HCl buffer, pH 8·0 containing 10 mM KCl, 5 mM β-mercaptoethanol and definite quantity of the effector was heated at various temperatures for 5 min, cooled in ice and assayed at 40°C.
(1), No addition; (2), 180 μM L-Valine; (3), 50 absorbance (260 nm) units of tRNA; (4), 50 absorbance (260 nm) units of tRNA plus 3 mM ATP.

insight into the thermal characteristics of the enzyme. The enzyme was heated at 50°C, the optimum growth temperature of the organism, with the substrates and spermine individually and in combination for various time intervals. Aliquots were withdrawn and assayed at 40°C as mentioned earlier. The results of these experiments confirmed the earlier finding that substrates could protect the enzyme against heat denaturation to varying degrees. When present together tRNA, ATP and spermine could impart complete protection for the duration of the incubation (figure 4).

Protein turnover

Rapid turnover of thermolabile macromolecules has been postulated as one of the mechanisms for growth at higher temperatures (Singleton and Amelunxen, 1973). In order to find out whether such a mechanism exists in *H. lanuginosa* the rate of protein turnover was studied by the double isotope method of Arias *et al.* (1969) as indicated under 'materials and methods'. The cells were grown at temperatures 40, 45, 50 and 55°C. The growth rate during the log phase at these temperatures was almost identical (data not presented). But the turnover rate was different at different temperatures. As the temperature of growth increased the protein turnover rate increased (table 1). This suggested that rapid turnover of proteins is also a mechanism operating in the fungus to overcome the ill effects of higher temperatures.

Discussion

Results of the present studies show that the tRNA of this fungus is thermostable up to the optimum temperature of its growth in the presence of small

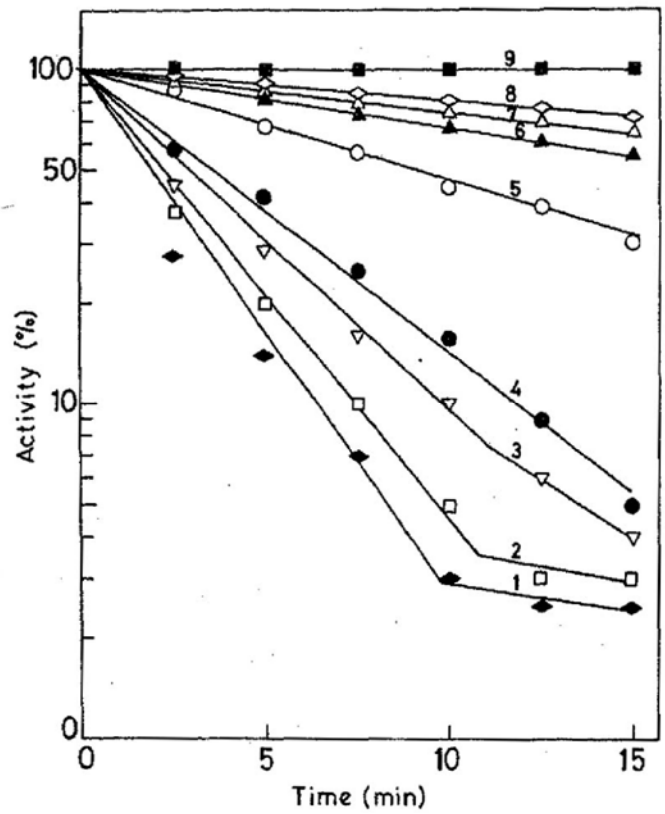

Figure 4. Rate of thermal denaturation of valine tRNA synthetase$_2$ at 50°C.

The enzyme was heated in the bufer at 50°C along with the effector and aliquots were withdrawn at definite time periods, chilled in ice and assayed at 40°C.
(1), No addition; (2), 180 μM L-Valine; (3), 0·25 mM spermine; (4), 3 mM ATP; (5), 180 μM L-Valine plus 3 mM ATP; (6), 50 absrobance (260 nm) units of tRNA; (7), tRNA plus 0·25 mM spermine; (8), tRNA plus 3 mM ATP; (9), tRNA plus 3 mM ATP plus 0·25 mM spermine.

Table 1. Protein turnover at different temperatures.

Growth temperature	[^{3}H]/[^{14}C]
40	1·34
45	1·34
50	2·1
55	4·7

H. lanuginosa cells were grown at a definite temperature and they were labelled with [^{14}C]-L-leucine for 30 min. After 2h chase in the cold medium the cells were again labelled, this time with [^{3}H]-leucine, for 10 min as described under 'methods'. The labelled cells were extracted with buffer and the radioactivity in an aliquot of the protein fraction was determined. [^{14}C]-and [^{3}H]-counts are expressed as a ratio.

quantities of $MgCl_2$ or spermine. Spermine has been reported to be present in biological systems. It is not known whether the fungus contains spermine. The experiments, however, suggest that the intracellular environment can alter the stability of the tRNA. No appreciable melting occurs upto 55°C. However, *H. lanuginosa* tRNA apparently has no special thermal stability features different from that of the mesophilic yeast, *C. utilis.* The fact that the tRNA of *H. lanuginosa* is more resistant to the action of RNase A (figure 1B) shows that it is more compact.

The preliminary results obtained with the total aminoacyl tRNA synthetases (figure 2) when viewed in the light of the thermal characteristics of purified valine tRNA synthetase 2 (figures 3 and 4) suggest that other enzymes also of this fungus may not be very thermostable. They require stabilisation *in vivo* by various cellular factors. The overall picture that emerges from this study is that, unlike thermophilic bacteria, the protein synthesizing machinery of if. *lanuginosa* in general does not possess the intrinsic thermostability required for growth at higher temperatures. It is stabilised by interaction with magnesium ions, ATP, tRNA, polyamines and other cellular factors. The increase in the rate of protein turnover with growth temperature (table 1) suggests that optimum levels of some of the proteins are maintained by this mechanism also. Thus it appears that the presence of intrinsically thermostable proteins is not very crucial for thermophilic growth in fungi. Unlike bacteria these organisms do not grow at very high temperatures. During evolution the requirement for highly thermostable macromolecules may not have been stringent as in the case of bacteria. Stability acquired through alterations in the microenvironment of the cell and interaction with protective agents and also to some extent increased turnover appear to play more important roles as mechanisms to counteract the detrimental effects of higher temperature in *H. lanuginosa.*

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