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Thermal stability of aminoacyl-tRNAs in aqueous solutions

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Abstract Life in hot environments poses certain constraints on the metabolism of thermophilic organisms. Many universal metabolic intermediates are quite labile compounds, and without protection will rapidly decompose at elevated temperatures. Among these are aminoacyl-tRNAs that are necessarily formed upon functioning of the translation apparatus. Aminoacyl-tRNAs are known to be hydrolyzed rapidly even at moderate temperatures under mild alkaline conditions. We studied the thermal stability of phenylalanyl- and alanyl-tRNA in aqueous solutions in order to evaluate a potential threat posed by high temperatures to these components of the translation machinery of thermophiles. Specific second-order rate constants of the aminoacyl-tRNA hydrolysis reaction were determined in the range 20°–80°C. The activation energy of phenylalanyl- and alanyl-tRNA hydrolysis was found to be about 42 and 23 kJ/mol, respectively. The calculated half-lives of aminoacyl-tRNAs at sub-80°C temperatures vary from several seconds to several dozens of seconds at near-neutral pH. The possible mechanisms counteracting the observed thermolability of aminoacyl-tRNAs in vivo are discussed.

Key words Aminoacyl-tRNA · Metabolism · Translation · Thermophiles · Thermostability

Introduction

Despite apparent variability, cellular metabolic networks of all living beings are built around a uniform set of the core biochemical reactions. Adaptation of the organisms to different environments may result in profound differences in cell morphology, motility, nutrient consumption and transformation, and so on, but does not change the nature of the basal processes that ensure DNA, RNA, and protein biosynthesis. Peripheric modules of the metabolic network, which are responsible for direct interaction with the immediate surroundings, can vary in type and number among species depending on their living conditions. At the same time, the core processes can only be adapted but not substituted. Therefore, the expansion of known life forms has its natural limits defined primarily by physicochemical constraints on stable functioning of these key metabolic pathways.

Temperature is one of the universal environmental characteristics. The observed thermal span of active life extends approximately from –10° to 110°C (Kristjansson and Stetter 1992). The upper temperature limit of growth is about 60°C for Eukarya and 95°C for Bacteria (Rothschild and Mancinelli 2001; Stetter 1999). The most extreme thermophiles known so far are representatives of the archaeal genera *Methanopyrus* and *Pyrodictium*, thriving at temperatures up to 110°C, among them *Pyrolobus fumarii*, which is able to grow even at 113°C (Stetter 1999; Blöchl et al. 1997). Until now it has been unclear which kinds of cellular components or processes set these limits for different groups of organisms and for life in general. For a long time it was thought that the intrinsic instability of proteins and nucleic acids represents one of the main barrier to the colonization of high-temperature habitats (Sterner and Liebl 2001; Scandurra et al. 1998; Jaenicke and Bohm 1998; Marguet and Forterre 1994; Grogan 1998; Kowalak et al. 1994). The lipid bilayer was regarded as another putative thermosensitive component of the cell (Tolner et al. 1997; Albers et al. 2000). As a result, a huge number of studies have been done in order to investigate the influence of high temperature on the structural properties of biologically important macromolecules and lipid membranes. At the same time, little attention has been paid to the thermal stability of the universal metabolic intermediates, some of them being very reactive (and, as a consequence, very labile) even at moderate temperatures (Daniel and Cowan 2000; Glansdorff 1999; White 1984). Thermodegradation of such intermediates may significantly decrease the output of the corre-

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sponding biochemical processes. Thermoinduced decay of the reactive metabolites may also result in uncontrolled side reactions that damage cellular constituents. In the present study we explore the thermal stability of aminoacyl-tRNAs that are necessarily formed in all known organisms upon functioning of the translation apparatus. The amino acid moiety of an aminoacyl-tRNA is attached to one of the hydroxy groups of the tRNA 3′-terminal adenosine via an ester bond, which is highly susceptible to a base-promoted hydrolysis. Here we analyze the temperature dependence of the hydrolytic decay of phenylalanyl- and alanyl-tRNAs in order to evaluate a potential threat posed by the aminoacyl-tRNA thermolability to proper functioning of the translation apparatus of thermophiles.

Materials and methods

Chemicals, tRNAs, and enzymes

L-[U-^{14}C]Phenylalanine (0.448 Ci/mmol) and L-[2,3-^{3}H] alanine (42.0 Ci/mmol) were obtained from Amersham Life Science, Na_2-ATP and spermine*4HCl from Sigma, and all other chemicals from Fluka. *Thermus thermophilus* $tRNA^{Phe}$ and $tRNA^{Ala}$ with amino acid acceptance 1,210 and 860 pmol/OD_{260} unit, respectively, were obtained according to the methods of Watanabe et al. (1980) and Bischoff and McLaughlin (1985). Phenylalanyl- and alanyl-tRNA synthetases were partially purified from *Thermus thermophilus* using techniques described by Ankilova et al. (1988) and Lechler et al. (1997).

pH measurements

pH measurements were performed with the use of an ORION Log R-compensated pH meter, which provides automatic temperature-corrected pH readings in the range 0°–100°C. Unless specifically mentioned, pH values were determined at room temperature.

Aminoacyl-tRNA synthesis

[^{14}C]Phenylalanyl-$tRNA^{Phe}$ was synthesized in a reaction mixture containing 5 mM ATP, 50 mM MOPS-NaOH (pH 7.0), 8 mM $MgCl_2$, 10 µM [^{14}C]Phe, 2 OD_{260} units/ml of $tRNA^{Phe}$ and 1.2 U/ml of phenylalanyl-tRNA synthetase. The reaction was carried out at 40°C for 2 h.

[^{3}H]Alanyl-$tRNA^{Ala}$ was synthesized in a reaction mixture containing 2.5 mM ATP, 100 mM HEPES-NaOH (pH 7.6), 10 mM $MgCl_2$, 0.5 mM spermine*4HCl, 1.5 µM [^{3}H]Ala, 3 OD_{260} units/ml of $tRNA^{Ala}$ and 1.0 U/ml of alanyl-tRNA synthetase. The reaction was carried out at 40°C for 1 h.

The tRNA aminoacylation reaction was stopped by the addition of sodium acetate (pH 5.0) to a final concentration 0.3 M, and the enzyme was removed by phenol extraction. Low-molecular-weight admixtures were washed away from aminoacyl-tRNA by repetitive buffer exchange with the use of a Microcon YM-10 membrane filter unit (Amicon). Then the aminoacyl-tRNA was precipitated from final buffer (0.3 M sodium acetate (pH 5.0)) by adding 3 volumes of ethanol. The precipitate was collected by centrifugation (10,000 *g*, 20 min, 4°C) and vacuum-dried. The aminoacyl-tRNA obtained was stored at –80°C.

Aminoacyl-tRNA hydrolysis

Deacylation of phenylalanyl-$tRNA^{Phe}$ was performed in a buffer containing 50 mM Tris-HCl and 1 mM Na_2-EDTA, with pH 8.96 at 25°C. Deacylation of alanyl-$tRNA^{Ala}$ was performed in a buffer containing 100 mM HEPES-NaOH, 6 mM Na_2-EDTA, with pH 7.66 at 25°C. The reactions were initiated by quick mixing of the buffer, preincubated for 15 min at the appropriate temperature, with a dry pellet of aminoacyl-tRNA. At predetermined times, aliquots were taken out from the reaction mixture and spotted onto Whatman 3MM paper filters impregnated with trichloroacetic acid (TCA). Then the filters were extensively washed with ice-cold 5% TCA to remove free amino acids, and TCA-insoluble radioactivity was determined by liquid scintillation counting. Deacylation rates were calculated from the time dependence of the residual amount of labeled amino acid bound to tRNA. The observed kinetics of aminoacyl-tRNA decay were characterized by a pseudo-first-order rate constant k_{app}.

Results

The general equation describing the alkaline hydrolysis of aminoacyl-tRNA ester bond can be expressed by:

$$\begin{aligned} &H_2N-CH(R)-C(O)\sim O-tRNA+OH \xrightarrow{k} \\ &\quad H_2-CH(R)-C(O)O^- + HO-tRNA \end{aligned} \tag{1}$$

The corresponding kinetic equation is:

$$\begin{aligned} d[aa\sim tRNA]/dt &= -k*[OH^-]*[aa\sim tRNA] \\ &= -k_{app}*[aa\sim tRNA] \end{aligned} \tag{2}$$

where [aa~tRNA] is the concentration of aminoacyl-tRNA, k is the second order rate constant of the reaction, and k_{app} is the apparent first-order rate constant, which depends on the concentration of hydroxyl anions in solution.

Temperature influences the observed rate of the aminoacyl-tRNA decay (k_{app}) by affecting the inherent susceptibility of the ester bond to alkaline hydrolysis, and the ionization state of buffer and water molecules that results in thermoinduced change of [OH^-]. That is to say, both k and [OH^-] are functions of temperature:

$$k_{app}(T) = k(T)*[OH^-]_T \tag{3}$$

Temperature dependence of the specific rate constant k can be expressed by Arrhenius' equation:

$$k(T) = k^{\#} * e^{-E_{act}/RT} \quad (4)$$

where $k^{\#}$ is a temperature-independent preexponential factor, E_{act} is the activation energy of the ester bond cleavage, and R is the gas constant.

The concentration of hydroxyl anions at each given temperature can be calculated from the experimentally determined pH of the solution and the tabulated values of the ionization constant of water, K_w:

$$\left[OH^-\right]_T = K_w(T) * 10^{pH(T)} \quad (5)$$

Thus, the dependence of k_{app} on temperature is expressed by:

$$k_{app}(T) = k^{\#} * e^{-E_{act}/RT} * K_w(T) * 10^{pH(T)} \quad (6)$$

or in logarithmic form:

$$\ln k_{app}(T) = -E_{act}/RT + pH(T) * \ln 10 + \ln K_w(T) + \ln k^{\#} \quad (7)$$

For convenience of computations, we approximated the temperature dependence of the ionization constant of water by an exponential function:

$$K_w(T) = K_w^{\#} * e^{-E_w/RT} \quad (8)$$

where $K^{\#}$ and E_w are constant, E_w is equal to 53.27 ± 0.51 kJ/mol, and $\ln K_w^{\#}$ is equal to –10.71 ± 0.20 at temperatures above 20°C.

Taking into account expression 8, Eq. 7 can be transformed to:

$$\ln k_{app}(T) = -(E_{act} + E_w)/RT + pH(T) * \ln 10 + \ln K_w^{\#} + \ln k^{\#} \quad (9)$$

Equation 9 allows us to determine the kinetic parameters of the ester bond cleavage, $k^{\#}$ and E_{act}, upon simultaneous variation in temperature and pH. A graph of the values $(\ln k_{app}(T) - pH(T) * \ln 10)$ versus $1/T$ should yield a straight line with the slope equal to $-(E_{act} + E_w)/R$ and the intercept, $\ln K_w^{\#} + \ln k^{\#}$.

In the above calculations we neglected the specific effect of the buffers on aminoacyl-tRNA deacylation and the temperature-induced changes of the ionization state of the α-amino group of an aminoacyl residue. Both these factors may contribute to the observed rates of aminoacyl-tRNA hydrolysis (Schuber and Pinck 1974a, b). However, to introduce them into the formal description of the hydrolysis reaction would require additional parameters that are largely unknown. On the other hand, if these secondary effects are really significant, they should reveal themselves in a nonlinear dependence of $(\ln k_{app}(T) - pH(T) * \ln 10)$ on $1/T$.

Deacylation of phenylalanyl- and alanyl-tRNAs has been studied in Tris-HCl/EDTA and HEPES-NaOH/EDTA buffers, respectively. Temperature-driven pH changes of both buffers were considered to be significant enough to be taken into account in $k^{\#}$ and E_{act} calculations (see Table 1). Hydrolytic decay of aminoacyl-tRNAs at all tested conditions could be described by perfect pseudo-first-order kinetics. The corresponding apparent rate constants (k_{app} values) were determined in the temperature range 20°–80°C (Table 1). Plots of $(\ln k_{app}(T) - pH(T) * \ln 10)$ against $1/T$ show little deviation from linearity (Fig. 1), which indicates an adequate choice of the model of the reaction mechanism. Fitting the experimental data sets into Eq. 9 yielded $\ln k^{\#}$ 25.00 ± 0.55 and E_{act} 42.63 ± 1.46 kJ/mol for phenylalanyl-tRNA hydrolysis, and $\ln k^{\#}$ 20.04 ± 1.10 and E_{act} 23.17 ± 2.99 kJ/mol for alanyl-tRNA hydrolysis.

Discussion

Upon mRNA-directed polypeptide chain synthesis on the ribosome, amino acids are conveyed to the reaction area in

Table 1. Apparent rate constants of the reaction of aminoacyl-tRNA hydrolysis

Temperature (°C)	Phe~tRNA^{Phe}		Ala~tRNA^{Ala}	
	(k_{app}, min^{-1})	pH	(k_{app}, min^{-1})	pH
20	0.0162 ± 0.0014	9.09		
30			0.0294 ± 0.0055	7.64
40	0.0570 ± 0.0035	8.56	0.0780 ± 0.0075	7.57
50			0.1604 ± 0.0216	7.49
60	0.2015 ± 0.007	8.11	0.3598 ± 0.0155	7.43
70			0.6141 ± 0.0136	7.37
80	0.5122 ± 0.0227	7.70	1.0220 ± 0.0463	7.32

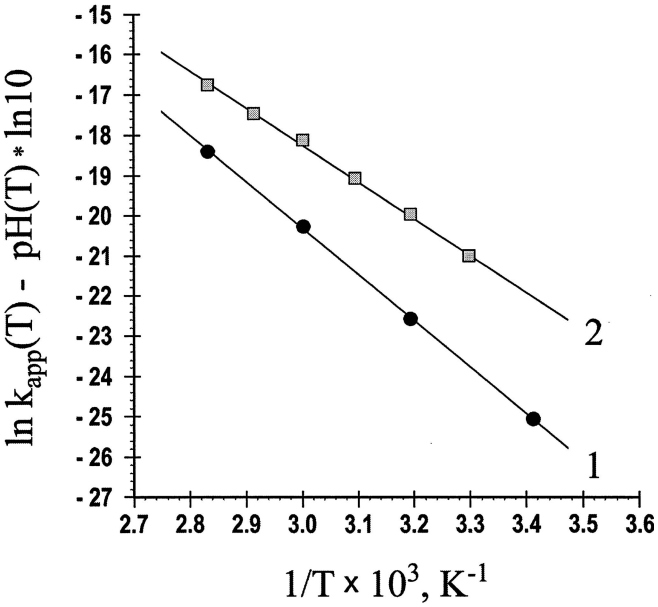

Fig. 1. Plot of $(\ln k_{app}(T) - pH(T) * \ln 10)$ against $1/T$ for the hydrolysis of phenylalanyl-tRNA (*1*) and alanyl-tRNA (*2*)

Table 2. Specific second-order rate constants of aminoacyl-tRNA hydrolysis calculated from the Arrhenius equation parametrized with E_{act} and $k^{\#}$ determined in this study

Temperature (°C)	Phe~tRNA^{Phe}		Ala~tRNA^{Ala}	
	(k, $M^{-1}{}_{*}\,min^{-1}$)	$k(T)/k(20°C)$	(k, $M^{-1}{}_{*}\,min^{-1}$)	$k(T)/k(20°C)$
0	506	0.28	18640	0.50
20	1823	1.00	37394	1.00
40	5571	3.06	68633	1.84
60	14889	8.17	117112	3.13
80	35597	19.53	188098	5.03
100	77514	42.52	287150	7.68

form of aminoacyl-tRNAs. An ester bond between an aminoacyl residue and the hydroxy group of the 3′-terminal adenosine of tRNA is relatively labile. A nucleophilic attack at the carbonyl carbon results in the split of the bond with subsequent transfer of the aminoacyl residue to the nucleophile. The reactivity of the aminoacyl-tRNAs is revealed by their quick decay in slightly alkaline aqueous solutions due to interaction with hydroxy anions. A high potential of acyl group transfer is a general property of esters, but in the case of aminoacyl-tRNAs it is additionally increased because of the negative induction effect of both the protonated α-amino group of the amino acid moiety and the vicinal *cis*-hydroxy group of the ribose (Jencks 1969; Söll and Schimmel 1974). The latter can also contribute to the aminoacyl-tRNA accelerated hydrolysis through stabilization of the transient state by hydrogen bonding (Bruice and Fife 1961, 1962). Stability of the ester bond also depends on the nature of the side chain of the amino acid but is not influenced by that of tRNA (Hentzen et al. 1972; Söll and Schimmel 1974).

Quantitatively, the susceptibility of aminoacyl-tRNA to alkaline hydrolysis can be expressed by the magnitude of the specific second-order rate constant k. Values of k, calculated on the basis of the Arrhenius equation, are presented in Table 2. Alanyl-tRNA has significantly lower stability than phenylalanyl-tRNA over the whole range of biologically relevant temperatures. This is in agreement with an observed tendency of alanyl-tRNA to decompose more rapidly than most other aminoacyl-tRNAs, including phenylalanyl-tRNA (Hentzen et al. 1972).

The intrinsic strength of the aminoacyl ester bond can be characterized by the activation energy (E_{act}) required to overcome the barrier of the hydrolytic reaction. The magnitude of the preexponential factor, $k^{\#}$, serves in a given context as a quantitative measure of orientational effects upon nucleophilic attack of the hydroxy anion on the carbonyl carbon. Comparison of the kinetic parameters of phenylalanyl-tRNA and alanyl-tRNA decay with those of the hydrolytic cleavage of the non-activated ester bond reveals $k^{\#}$ as a component, which essentially contributes to the elevated lability of both aminoacyl-tRNAs (Table 3). In the case of alanyl-tRNA, the ester bond splitting is further accelerated because of a relatively low E_{act}. The increased values of the preexponential factor $k^{\#}$ for the aminoacyl-tRNA hydrolysis may be caused by involvement of the *cis*-vicinal hydroxy group of the tRNA 3′-terminal ribose in the favorable positioning of the attacking hydroxy anion and/or in the formation of the hydrogen bond to an oxygen atom of the ester in the transient state (the so-called "internal solvation effect") (Bruice and Fife 1961, 1962). The magnitude of E_{act} determines the extent of change of k upon elevation of temperature. Consequently, the rate of hydrolysis of phenylalanyl-tRNA is more strongly influenced by temperature than that of alanyl-tRNA (Table 2). The increase of temperature is accompanied by a decrease of difference between the stabilities of these two aminoacyl-tRNAs.

Both phenylalanyl- and alanyl-tRNA should have quite short half-lives at sub-80°C temperatures, optimal for the growth of many thermophiles (Table 4). They are more thermolabile than some other important metabolic intermediates, including such energy-rich compounds as ATP, acetyl-CoA, and phosphorylated derivatives of glucose and ribose (Daniel and Cowan 2000; Oro et al. 1990). This finding immediately raises a question about the possible mech-

Table 3. Parameters of the Arrhenius equation for alkaline hydrolysis of aminoacyl-tRNAs and selected aliphatic esters

Compound	E_{act} (kJ/mol)	ln $k^{\#}$
Alanyl-tRNA	23.17	20.04
Phenylalanyl-tRNA	42.63	25.00
Ethyl acetate	47.7[a]; 49.3[b]	17.03[a]; 17.68[b]
Ethyl propionate	44.8[c]; 44.3[b]	15.61[c]; 15.45[b]
Ethyl butyrate	43.2[c]; 44.6[b]	14.14[c]; 14.94[b]

[a] Tommila and Murto (1963)
[b] Halonen (1956)
[c] Tommila and Hietala (1954)

Table 4. Half-lives ($\tau_{1/2}$) of aminoacyl-tRNAs at pH 7.5 calculated from the corresponding specific second-order rate constants (k) according to the equation $\tau_{1/2} = \ln 2/(k(T)*[OH^-]\tau)$

Temperature (°C)	Phe~tRNA^{Phe}	Ala~tRNA^{Ala}
0	26.4 days	17.2 h
20	29.4 h	1.4 h
40	2.2 h	10.9 min
60	15.3 min	1.9 min
80	2.4 min	27 s
100	29 s	8 s

anisms of counteraction of the accelerated degradation of aminoacyl-tRNAs at elevated temperatures.

It is well known that aminoacylated elongator tRNAs can be efficiently protected against hydrolysis by being part of the ternary complex with translation elongation factor (EF-Tu in case of Bacteria or EF-1α in case of Archaea and Eukarya) and GTP (Krab and Parmeggiani 1998). The amino group of the aminoacyl residue is likely to be deprotonated when aminoacyl-tRNA is bound to the elongation factor. As a result, the aminoacyl ester bond becomes less susceptible to nucleophilic attack due to the decreased electrophilicity of the carbonyl carbon atom. Furthermore, being buried into the protein-binding cleft, the ester bond is hardly accessible to the hydroxy anions and other nucleophiles (Nissen et al. 1999). The magnitude of the dissociation constant of the ternary complex in vivo at elevated temperatures is quite uncertain. Thermodynamic stability of the ternary complex was characterized generally in vitro at near 0°C. At these conditions the dissociation constants were found to be in the nanomolar range for bacterial EF-Tus and in the micromolar range for archaeal EF-1αs (Louie and Jurnak 1985; LaRiviere et al. 2001; Abrahamson et al. 1985; Ott et al. 1989; Raimo et al. 2000). Therefore, inside the cell living at moderate temperatures, the bulk of aminoacylated elongator tRNAs should be trapped in a complex with the elongation factor, taking into account the vast abundance of this protein in the cytoplasm (0.1–0.2 mM). An elevation of the ambient temperature may cause a drastic increase in the dissociation constant of the ternary complex to the extent that an efficient protection of the whole aminoacyl-tRNA pool against hydrolysis is impossible (Vorstenbosch et al. 2000). On the other hand, the crowded intracellular environment strongly favors the association of macromolecules and can compensate the observed in vitro temperature-induced decrease of the ternary complex stability (Ellis 2001). Thus, a substantial amount of aminoacyl-tRNA in vivo could still be kept in complex with the elongation factor even at high temperatures. Here we would like to mention that the protein not only protects aminoacyl-tRNAs from hydrolysis but also prevents undesirable side reactions of the activated amino acid with other cellular components. Such isolation of highly reactive metabolic intermediates from direct contact with intracellular environment by trapping them in a complex with protein could be a widespread strategy among thermophiles. Another very labile metabolite, carbamoyl phosphate (half-life less than 2 s at 100°C), is likely to be protected from thermodegradation in a similar way in *Pyrococcus furiosus* (Massant et al. 2002).

Influence of the thermal instability of aminoacyl-tRNAs on the proper functioning of the translation apparatus will be reduced if aminoacyl-tRNA turnover proceeds quickly. In *Escherichia coli* cells at 37°C the turnover of individual aminoacyl-tRNAs varies from 1.7 to 8.1 s^{-1} (Jakubowski and Goldman 1984). This means that even unprotected aminoacyl-tRNA would decompose very slowly compared to its average lifetime (0.12–0.6 s) from synthesis by aminoacyl-tRNA synthetase and to consumption by the ribosome. In other words, for any aminoacyl-tRNA molecule the probability of being unproductively hydrolyzed is much lower than the probability to bind to the ribosome and to be used for polypeptide chain elongation. In a similar way, thermophilic organisms may overcome the aminoacyl-tRNA thermolability problem by increasing both the rate of polypeptide synthesis on the ribosome and the activity of aminoacyl-tRNA synthetases. Unfortunately, little is known about the rate of polypeptide chain elongation in thermophiles at their optimal growth conditions. The moderately thermophilic bacterium *Bacillus stearothermophilus* ($T_{optimal}$ 50°C) was observed to have significantly faster renewal of the intracellular proteins than mesophilic *E. coli* (Bubela and Holdsworth 1966a, b). However, a high rate of protein synthesis tends to be more an attribute of all *Bacillus* species (including the mesophilic ones) than a result of *B. stearothermophilus* adaptation to a hot environment (Amelunxen and Murdock 1978). Another well-studied thermophile, *Thermus thermophilus* ($T_{optimal}$ 75°C), was reported to have a rate of protein synthesis comparable with that of *E. coli* (Ohno-Iwashita et al. 1975). At the same time, the specific activity of *Th. thermophilus* phenylalanyl-tRNA synthetase measured in vitro at the optimal growth temperature is higher than that of the *E. coli* enzyme (Ankilova et al. 1988; Stepanov et al. 1998; Bobkova et al. 1992). Therefore, the aminoacyl-tRNA turnover in *Th. thermophilus* cells at 75°C is likely to proceed at the same speed as in *E. coli* cells at 37°C, but the faster aminoacyl-tRNAs decay is compensated for by their faster resynthesis by aminoacyl-tRNA synthetases. No experiments were performed with hyperthermophilic Archaea living at 100°–110°C, whose extreme position on the temperature scale of life raises many interesting questions about the peculiarities of the function of their metabolic chains. Thus, at present it is difficult to say definitively whether thermophiles utilize the above-mentioned strategy to circumvent aminoacyl-tRNA instability at near-boiling temperatures.

In conclusion, it is important to note that despite the observed thermolability of aminoacyl-tRNAs in vitro, thermophilic organisms can rely on simple but efficient mechanisms to escape its negative consequences in vivo. The highly reactive aminoacyl ester bond can be protected from the nucleophile-rich intracellular environment by the formation of a complex between aminoacyl-tRNA and elongation factor and thus will be stabilized. The quick turnover of aminoacyl-tRNAs makes the protein synthesis machinery less sensitive to the instability of the aminoacyl ester bond. An increased activity of aminoacyl-tRNA synthetases can compensate for the faster decay of aminoacyl-tRNAs at high temperatures.

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