Effect of Temperature on Stability and Activity of Elongation Factor 2 Proteins from Antarctic and Thermophilic Methanogens

TORSTEN THOMAS AND RICARDO CAVICCHIOLI*

School of Microbiology and Immunology, The University of New South Wales, Sydney 2052, NSW, Australia

Received 21 September 1999/Accepted 9 December 1999

Despite the presence and abundance of archaea in low-temperature environments, little information is available regarding their physiological and biochemical properties. In order to investigate the adaptation of archaeal proteins to low temperatures, we purified and characterized the elongation factor 2 (EF-2) protein from the Antarctic methanogen Methanothrix marisvitea, which was expressed in Escherichia coli, and compared it to the recombinant EF-2 protein from a phylogenetically related thermophile, Methanosarcina thermophila. Using differential scanning calorimetry to assess protein stability and enzyme assays for the intrinsic GTPase activity, we identified biochemical and biophysical properties that are characteristic of the cold-adapted protein. This includes a higher activity at low temperatures caused by a decrease of the activation energy necessary for GTP hydrolysis and a decreased activation energy for the irreversible denaturation of the protein, which indicates a less thermostable structure. Comparison of the in vitro properties of the proteins with the physiological characteristics of growth of the organisms indicates that additional cytoplasmic factors are likely to be important for the complete thermal adaptation of the proteins in vivo. This is the first study to address thermal adaptation of proteins from a free-living, cold-adapted archaeon, and our results indicate that the ability of the Antarctic methanogen to adapt to the cold is likely to involve protein structural changes.

It is now clearly established that archaea are present in low-temperature environments and not restricted to extreme environments such as high-temperature and high-salt habitats (7). In regions of the ocean, archaea have been reported to contribute up to 34% of the prokaryotic biomass (8). While this implies that archaea have a significant ecological role, little information is available concerning physiological or biochemical properties of these organisms in these cold habitats. This is largely due to the difficulties in isolating low-temperature-adapted (psychrophilic or psychrotolerant) archaea from the environment and cultivating them in the laboratory. Franzmann and colleagues have, however, successfully isolated and described monocultures of three archaeal organisms from Arctic lakes (12). One of these, Methanothrix marisvitea, was isolated from the anaerobic, methane-saturated, bottom waters of Ace Lake, where the temperature is a constant 1 to 2°C (13). Methanosarcina thermophila, a methanogenic archaeon and has a growth temperature range from −2.5 to 28°C, with fastest growth occurring at 23°C.

The mechanisms allowing psychrophilic and psychrotolerant (11, 24, 25) and mesophilic (18, 19, 25, 30) bacteria and eukaryotes to adapt to low temperatures have been reviewed elsewhere. Organisms growing at low temperatures encounter a number of growth constraints: enzyme reaction rates decrease, the affinity of uptake and transport systems decreases, membranes become less fluid, and nucleic acid structures become less thermostable. In response, microorganisms have evolved various ways to adapt. For example, increases in membrane fluidity are obtained through a relative increase in polyunsaturated fatty acids, and microorganisms that are restricted to temperature ranges below 15 to 20°C tend to be found in environments that are rich in organic substrates to compensate for their lesser effective uptake and transport systems. Cold shock and cold acclimation proteins are also synthesized to enable gene expression to continue at low temperatures.

Psychrophilic and psychrotolerant bacteria and eukaryotes appear to compensate for the limitations imposed by reduced thermal energy by producing proteins with a higher specific activity at low temperatures than that of their mesophilic or thermophilic counterparts (15). The increased activity at low temperatures is thought to be due to a higher flexibility of protein structure. As a consequence, cold-adapted proteins are also less thermostable. A number of structural features have been identified as contributing to a less stable or more flexible structure, including the loss of salt bridges, greater solvent interaction with surface structures, and extended loop structures (reviewed in reference 11).

We have recently reported a structural and evolutionary analysis of the archaeal elongation factor 2 (EF-2) proteins from Methanosarcina thermophila and closely related mesophilic and thermophilic methanogens (31). EF-2 is a GTPase involved in the translocation step of the ribosome during protein synthesis. One of the most significant differences between mesophilic and cold-adapted bacteria is that ribosomes remain active at low temperatures (2, 3, 5, 17, 23, 33). While comparative studies have not been performed with archaea, due to the essential function of protein synthesis, it is likely that ribosomes and associated factors are also thermally adapted. Comparison of the predicted three-dimensional structure of the EF-2 proteins of Methanosarcina thermophila (fastest growth at 50 to 55°C), has shown that the Methanosarcina thermophila EF-2 possesses structural features indicative of a more flexible (unstable) protein (31). These features include fewer salt bridges, less-packed hydrophobic cores, and the reduction of proline residues in loop structures. As a result, it is expected that the Methanosarcina thermophila EF-2 will have lower stability and increased activity at low temperatures.

In this study, we present a comparative biochemical and
biophysical characterization of the EF-2 proteins from *M. burtonii* and *M. thermophila*. The proteins were overexpressed in *Escherichia coli* and purified to homogeneity. By using differential scanning calorimetry (DSC), the *M. burtonii* EF-2 protein was shown to have lower thermostability than the *M. thermophila* EF-2. By in vitro GTase assays, the *M. burtonii* EF-2 was also shown to possess a higher intrinsic GTase activity at low temperatures. Moreover, it has been found that the activity and stability profiles of the proteins did not simply correlate with the temperatures at which each methanogen had the highest growth rate. The implications of these findings are discussed.

**MATERIALS AND METHODS**

**Construction of expression vector.** Genomic DNA was extracted and purified from *M. burtonii* and *M. thermophila* as described previously (11). The 2F genes (accession no. AF003869 and AF022779 for *M. burtonii* and *M. thermophila*, respectively) were cloned into the expression vector pCYB2 (New England Biolabs) according to the method of Tillett and Neilan (32), enabling the expression of the genes without additional vector-derived amino acid residues. The recombinant constructs encoded the self-cleavable intein from *Saccharomyces cerevisiae* and a chitin-binding domain fused to the carboxyl terminus of EF-2. To construct the recombiant plasmids, the genes from each organism were PCR amplified and purified using different sets of primers. For the first amplification, the primers were 5' TAGGATCCGGCAAGAGGAAAGGAAATGTTGAGGCGGTTGTTGGTGAAGTG-3' and MB3S (5'-AGCACAAGAGTTGGAGGCGGTTG3'). For the second amplification, the primers were 5'-AAAGAAGGCTGCGGAGGACCAAGACATGCTCATAAAGGTAACACGCACGATGCTAATAAGTTTAAAGGCGAGGATTGAAGTTCAAGGTTTGTTGGTGAAGTG-3' and MB5L (5'-GGTACCGGAGGCGGTTG3').

**Overexpression and purification.** For the first amplification, the primers were ESM (5'-AAGAAAAGGCTGAGGAGGACCAAGACATGCTCATAAAGGTAACACGCACGATGCTAATAAGTTTAAAGGCGAGGATTGAAGTG-3') and MB3L (5'-GGTACCGGAGGCGGTTG3'). The second amplification, the primers were 5'-CCTTCGTCCCATATGCTATGCTAATAAGTTTAAAGGCGAGGATTGAAGTG-3' and MB5L (5'-GGTACCGGAGGCGGTTG3'). The vector pcYB2 was also amplified in two PCRs containing the primers VSLM (5'-CCTCGTCCGCAAGTTTAAAGGCGAGGATTGAAGTG-3') and VSS (5'-AATTTTGTTTAAAGGCGAGGATTGAAGTG-3'), and V5L (5'-TGGCTTCTGGTTTAAAGGCGAGGATTGAAGTG-3') and V3L (5'-TGGCTTCTGGTTTAAAGGCGAGGATTGAAGTG-3'). For the vector, reactions were carried out in 20-μl volumes with 100 ng of genomic DNA, 10 pmol each of primer, 2.75 mM MgCl2, Taq reaction buffer (Boehringer Mannheim), and 1 U of Taq (Boehringer Mannheim)-PFU (Stratagene) polymerase mix (unit ratio, 1:10) for 25 cycles (95°C, 10 s; 55°C, 20 s; 72°C, 4 min) after initial denaturation for 2 min at 95°C. The vector pcYB2 was also amplified after initial denaturation for 2 min at 95°C. The vector pCYB2 was also amplified in two PCRs containing the primers VSLM (5'-CCTCGTCCGCAAGTTTAAAGGCGAGGATTGAAGTG-3') and VSS (5'-AATTTTGTTTAAAGGCGAGGATTGAAGTG-3'), and V5L (5'-TGGCTTCTGGTTTAAAGGCGAGGATTGAAGTG-3') and V3L (5'-TGGCTTCTGGTTTAAAGGCGAGGATTGAAGTG-3'). For the vector, reactions were carried out in 20-μl volumes with 10 ng of vector, 10 pmol of each primer, 2.75 mM MgCl2, Taq reaction buffer (Boehringer Mannheim), and 1 U of Taq (Boehringer Mannheim)-PFU (Stratagene) polymerase mix (unit ratio, 1:10) for 25 cycles (95°C, 10 s; 55°C, 20 s; 72°C, 4 min) after initial denaturation for 2 min at 95°C. All amplicons were purified with a Prep-a-gene kit (Bio-Rad) and dialyzed against 20 mM morpholinepropanesulfonic acid (MOPS) (pH 7.5) and 1 mM β-mercaptoethanol. The final dialyze was kept as a reference for the DSC. Prior to loading, the samples were filtered (0.2-μm-pore-size filter) and desalted with stirring for 10 min at 4°C. Calorimetry was performed on a MicroCal VP-DSC calorimeter, and data were analyzed with the Origin MicroCal DSC software package.

**RESULTS AND DISCUSSION**

**Overexpression and purification of the EF-2 proteins.** In order to investigate the biochemical and biophysical properties of the EF-2 proteins from *M. burtonii* and *M. thermophila*, both proteins were overexpressed in *E. coli* as fusion proteins with a yeast intein and a chitin-binding domain. This system has been successfully applied to the expression and purification of another archaeal protein (27). In *E. coli* BL21, expression levels of both fusion proteins were low, with yields estimated at 0.1 to 0.2 mg from pMB and 0.4 to 0.5 mg from pMT for fusion proteins per g of cell wet weight (cw) (Fig. 1). The *M. burtonii* and *M. thermophila* aef-2 genes, respectively, were cloned into the expression vector pCYB2, which encodes the E. coli RNAaseT2 and a chitin-binding domain. The upper arrowhead (F) indicates the position of the EF-2 fusion protein; the lower arrowhead (E) indicates the position of the EF-2 protein.
elongation factor protein from an archaeal hyperthermophile (9).

The purified proteins from *M. burtonii* and *M. thermophila* had a molecular mass as determined by mass spectrometry of 80,567 and 80,631 (±100) Da, respectively, which correlates well with the theoretical masses of 80,477 and 80,564 Da, respectively, derived from the amino acid sequences (31).

Protein stability and thermal unfolding. The thermostability of the purified proteins was examined by DSC. At scan rates (*v*) of 1.5 K/min, the temperature values of the maximum heat capacity (*T_m*) were 50.5 and 55.6°C for *M. burtonii* and *M. thermophila* EF-2, respectively (Fig. 2A). Rescanning of the protein samples after they were heated beyond the transition peak (i.e., 55 to 60°C) and then cooled to 4°C resulted in no further increases in heat capacity (data not shown). This indicates that the unfolding of the EF-2 proteins from both organisms is an irreversible process.

When the scan rate was decreased (*v* = 0.1 K/min), the values for *T_m* shifted toward lower temperature values with 37.4 and 49.7°C observed for the *M. burtonii* and *M. thermophila* EF-2, respectively (Fig. 2B). Significant changes in the shape of the heat capacity curve were also observed. It has previously been shown that the shape of DSC thermograms of irreversible processes is scan rate dependent (26). In order to further analyze the denaturation process, a simple kinetic model for the thermal transition of the EF-2 proteins was used, where the native protein *N* undergoes an endothermic and irreversible step to a denatured state *D* with a first-order rate constant *k* (*N* → *D*). According to this model, the rate constant of the reaction at a given temperature *t* can be calculated by the formula *k* = *vC_p*(Q – *Q_t*) with *C_p* being the excess heat capacity at *t*, *Q_t* being the heat evolved at a given *t*, and *Q* being the total heat of the process (26).

The calculated values of *k* (as ln(*k*)) for the *M. burtonii* and *M. thermophila* EF-2 proteins for three different scan rates (*v* = 0.1, 0.5, and 1.5 K/min) were plotted against the inverse of the absolute temperature (in K) (Fig. 3). According to the Arrhenius equation [*k* = *Ae^(−E/RT)*], the slope of the straight line corresponds to −*E/R*, with *E* being the activation energy of the process and *R* being the universal gas constant. From this, values for *E* of 203 and 351 kJ/mol were calculated for *M. burtonii* and *M. thermophila* EF-2, respectively. These data show that the activation energy for the unfolding process of EF-2 from *M. burtonii* is significantly lower than that for *M. thermophila* and indicate that the *M. burtonii* EF-2 has a less stable structure.

It should be noted that the model applied to analyze the thermograms may only represent an approximate description of the EF-2 unfolding and denaturation process. Deconvolution of the thermograms using various models incorporated in the Origin software (see description in Materials and Methods) indicated that the thermal unfolding of both proteins is best described by a model with three or more separate, non-two-phase unfolding events. This is consistent with the known multidomain structure of the bacterial homologue to EF-2, elongation factor G (1, 6), and the predicted structure of EF-2 (31). The model presented above, however, is sufficient to make a qualitative interpretation about the overall thermolability of the EF-2 proteins and to let us reach the conclusion that the *M. burtonii* protein is less stable.

![FIG. 2](http://jb.asm.org/) Excess heat capacity of the EF-2 proteins from *M. burtonii* (solid lines) and *M. thermophila* (dotted lines) versus temperature at scan rates of 1.5 (A) and 0.1 (B) K/min. Measurements were performed in 20 mM MOPS (pH 7.5) and 1 mM β-mercaptoethanol.

![FIG. 3](http://jb.asm.org/) Arrhenius plot for the reaction rate of thermal denaturation (calculated as described in the text) for *M. burtonii* EF-2 (circles) and *M. thermophila* EF-2 (squares). Values for three different scan rates (0.1, 0.5, and 1.5 K/min) were used. The straight lines represent linear fits to the data, which were used to calculate the activation energy of the reaction.
In vitro activity. The EF-2 proteins from both organisms showed a low intrinsic activity for the hydrolysis of GTP to GDP. For example, 0.02 mol of GTP was hydrolyzed per mol of *M. thermophila* EF-2 per min at 40°C. This is comparable to the activity in the absence of stimulating factors described for the EF-2 protein from the archaeal hyperthermophile *Sulfolobus solfataricus* (0.016 mol of GTP hydrolyzed per mol of *S. solfataricus* EF-2 per min at 60°C) (20).

The intrinsic activity of *S. solfataricus* EF-2 has been shown to be stimulated about 300-fold by the presence of aliphatic alcohols and divalent cations. This stimulatory effect was attributed to the increased affinity of the EF-2 for GTP and was proposed to involve a conformational change in a hydrophobic region near the catalytic site (21). The effect of various combinations of aliphatic alcohols and divalent cations was tested with the EF-2 proteins from *M. burtonii* and *M. thermophila*. The highest level of stimulation (about eightfold) for both proteins was observed by the addition of 10 mM BaCl$_2$ and 10% (vol/vol) 2-propanol. As a result, these conditions were used for subsequent activity assays. These conditions are similar to those used for the *S. solfataricus* EF-2 (greatest stimulation by 8 mM BaCl$_2$ and 40% ethylene glycol) and *E. coli* elongation factor G (16-fold stimulation by 20% 2-propanol) (10).

In the GTPase assays, the amount of GTP hydrolysis was directly proportional to the amount of purified EF-2 added, thereby indicating that the reaction observed is enzymatically catalyzed. When a maltose-binding protein was purified by the same procedures, the GTP hydrolysis observed was equivalent to that observed for spontaneous GTP hydrolysis (data not shown). This demonstrates that the activity in assays containing the EF-2 proteins is not a result of contaminating enzymes.

Initial rates were used to determine the temperature-dependent, specific activity of both elongation factors. Marginal differences in temperature optima for activity (34 and 36°C) and maximum reaction rates (0.14 to 0.18 mol of GTP hydrolyzed per mol of EF-2 per min) were observed for EF-2 proteins from *M. burtonii* and *M. thermophila*, respectively (Fig. 4A). While the activity profiles for the proteins are similar, the loss of activity occurs at a lower temperature for the *M. burtonii* EF-2. In addition, the *M. burtonii* EF-2 has measurable activity at 7°C, whereas the lowest temperature for which activity was observed for the *M. thermophila* EF-2 was 21°C.

From the linear range of the Arrhenius plot, the activation energy for GTP hydrolysis was determined to be 35.5 and 70.7 kJ/mol for *M. burtonii* and *M. thermophila* EF-2, respectively (Fig. 4B). The activation energy for GTP hydrolysis of EF-2 from the moderate hyperthermophile *S. solfataricus* (fastest growth at 70 to 80°C), is 85 kJ/mol (20). These data for activation energy are consistent with the thermal energy of the environments in which the archaea grow and clearly demonstrate that the EF-2 proteins have undergone adaptations to enable catalytic activity under different thermal constraints.

Correlation of in vitro stability and activity with cellular physiology. The in vitro activity and stability profiles of the proteins were examined with respect to the upper temperature limits of growth of the organism and the temperature at which the organism has the highest growth rate. The *M. thermophila* EF-2 shows no activity and a partial unfolding of the protein at the temperature at which the organism has maximal growth rate (50 to 55°C). This may indicate that the in vitro assay conditions do not reflect physiological conditions in vivo and that intracellular factors may be important for stabilizing the protein. *M. thermophila* is known to produce and accumulate small, highly water-soluble molecules called compatible solutes in response to hyperosmotic conditions (29). Compatible solutes have been shown to stabilize proteins against heat stress (14, 16, 28) and may therefore play a role in thermal stabilization of the *M. thermophila* EF-2 protein in vivo.

The *M. burtonii* EF-2 shows its maximal in vitro activity (34°C) above the maximal growth temperature of the organism (28°C). Similar patterns of activity in comparison to temperature ranges of low-temperature-adapted microorganisms have frequently been observed (11). It is possible that the cytoplasm

![FIG. 4. (A) Specific activity of the GTP hydrolysis for *M. burtonii* EF-2 (circles) and *M. thermophila* EF-2 (squares) versus temperature. (B) Arrhenius plot showing the logarithm of GTP hydrolysis for *M. burtonii* EF-2 (circles) and *M. thermophila* EF-2 (squares) versus the reciprocal of the absolute temperature. Lines represent the regression of the linear range used to determine the activation energy of catalysis (for values, see the text). Activity was measured in the presence of 10% propanol and 10 mM BaCl$_2$.](http://jb.asm.org/Downloaded/from/http://jb.asm.org)
of the cold-adapted methanogen (and possibly of bacteria) contains factors that increase flexibility of the protein, thereby augmenting activity at low temperatures. Furthermore, EF-2 binds in vivo to rRNA and ribosomal proteins, and these interactions might modulate the temperature-dependent activity profiles of the EF-2 proteins.

These studies, by comparing the in vitro activity and stability properties of the EF-2 proteins from *M. burtonii* and *M. ther-mophila*, show that the *M. burtonii* protein possesses characteristics of a low-temperature-adapted protein. The in vitro characteristics, however, do not simply correlate with the maximal growth rates. In future studies, we will focus on the effects of intracellular components on in vitro activity and stability in order to gain a more complete understanding of the mechanisms of physiological adaptation to the cold.

ACKNOWLEDGMENTS

We thank Anne Poljak, Charles Gerday, Thierry Lohienne, Paul March, Ralf Mattes, and Daniel Tillett for help and advice and Paul Curmi and Staffan Kjelleberg for critical review of the manuscript.

This work was supported by the Australian Research Council, Large Grants scheme.

REFERENCES