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Thermodynamic activation properties of elongation factor 2 (EF-2) proteins from psychrotolerant and thermophilic Archaea

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Abstract In this study, the thermodynamic activation parameters of cold-adapted proteins from Archaea are described for the first time for the irreversible protein unfolding and ribosome-dependent GTPase activity of elongation factor 2 (EF-2) from the psychrotolerant *Methanococcoides burtonii* and the thermophilic *Methanosarcina thermophila*. Thermolability of *Methanococcoides burtonii* EF-2 was demonstrated by a low activation free-energy of unfolding as a result of low activation-enthalpy. Although structural data for EF-2 are presently limited to protein homology modeling, the observed thermodynamic properties are consistent with a low number of noncovalent bonds or an altered solvent interaction, causing a loss of entropy during the unfolding process. A physiological concentration of potassium aspartate or potassium glutamate was shown to stabilize both proteins against irreversible denaturation by strengthening noncovalent interactions, as indicated by increased activation enthalpies. The transition state of GTPase activity for *Methanococcoides burtonii* EF-2 was characterized by a lower activation enthalpy than for *Methanosarcina thermophila* EF-2. The relative entropy changes could be explained by differential displacement of water molecules during catalysis, resulting in similar activation free energies for both proteins. The presence of solutes was shown to facilitate the breaking of enthalpy-driven interactions and structuring of more water molecules during the reaction. By studying the thermodynamic activation parameters of both GTPase activity and unfolding and examining the effects of intracellular solutes and partner proteins (ribosomes), we were able to identify enthalpic and entropic properties that have evolved in the archaeal EF-2 proteins to enable *Methanococcoides burtonii* and *Methanosarcina thermophila* to adapt to their respective thermal environments.

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Introduction

As an abiotic extreme, low temperature constrains cellular activity in a number of important ways, including lowering enzyme reaction and solute uptake rates, reducing membrane fluidity, and stabilizing inhibitory nucleic acid structures (Cavicchioli et al. 2000; Russell 2000). Despite this, microorganisms are abundant in low-temperature environments, and a broad range of species from all domains of life have been identified from diverse cold habitats (Ravenschlag et al. 1999; Staley and Gossink 1999; Takami et al. 1999; Carpenter et al. 2000; Maruyama et al. 2000; Price 2000; Skidmore et al. 2000; Brambilla et al. 2001). A number of recent reports have identified a diversity and abundance of Archaea in low-temperature aquatic environments (Bowman et al. 2000; Ouverney and Fuhrman 2000; Massana et al. 2000; Karner et al. 2001), and a small number of free-living (Franzmann et al. 1988, 1992, 1997) or symbiotic species (Preston et al. 1996) have been isolated. Despite these findings, little information is available regarding the molecular and biochemical adaptation of these Archaea to low temperature (Cavicchioli et al. 2000).

In view of the considerable effect temperature has on protein activity and stability, a high proportion of studies examining cellular adaptation to low temperature have focused on the structural, biochemical, and biophysical properties of proteins. The main characteristics of enzymes from cold-adapted organisms are their superior ability to perform catalysis at low temperature and their sensitivity to thermal denaturation in comparison to equivalent proteins from mesophiles or thermophiles (reviewed in Feller et al. 1996; Gerday et al. 1997; Russell 2000). In association with these studies, a range of biochemical and structural characteristics that may account for the thermal properties of cold-active proteins have been identified.

Comparing the thermodynamic parameters of activation for proteins from cold-adapted and thermophilic organisms provides specific information about the relationship between protein structure and enzyme catalysis (by measuring activity) and between protein structure and enzyme stability (by measuring unfolding). In particular, the contributions that entropy (ΔS^*) and enthalpy (ΔH^*) make toward the overall free energy (ΔG^*) of activation can be calculated. For cold-adapted enzymes, enhanced catalysis at low temperature has been attributed to a reduction in ΔH^*; this is linked to the high structural flexibility that reduces the number of enthalpy-driven interactions that need to be broken during activation (Gerday et al. 1997; Lonhienne et al. 2000). The high structural flexibility may also cause a decrease in ΔS^* with a concomitant decrease in the rate of reaction. There is little information available regarding the thermodynamic activation parameters of denaturation of cold-active proteins (Ciardiello et al. 2000). Conceivably, however, thermolability may be achieved enthalpically through a decrease in ΔH^* (Violet and Meunier 1989; Rashid and Siddiqui 1998) or entropically through an increase in ΔS^* (Ciardiello et al. 2000).

We have examined the structural properties of elongation factor 2 (EF-2) from psychrotolerant (*Methanococcoides burtonii*), mesophilic (*Methanococcoides methylutens*), and thermophilic (*Methanosarcina thermophila*) Archaea (Thomas and Cavicchioli 1998) and studied the temperature dependence of GTPase activity and protein denaturation (Thomas and Cavicchioli 2000). Recently, we examined the effects of ribosomes and intracellular solutes on the activity and stability of EF-2 proteins from *Methanococcoides burtonii* and *Methanosarcina thermophila* (Thomas et al. 2001). Ribosomes greatly stimulated the GTPase activity of both proteins. In contrast, each organism produced a distinct combination of solutes, and those tested (potassium aspartate from *Methanococcoides burtonii* and potassium glutamate from *Methanosarcina thermophila*) had specific effects on catalysis and protein stability. In the present study, we calculated the activation parameters of ribosome-dependent guanosine triphosphate (GTP) hydrolysis and thermal unfolding for EF-2 proteins from *Methanococcoides burtonii* and *Methanosarcina thermophila*. The comparative thermodynamic activation parameters provided a useful means of assessing how the EF-2 proteins have evolved in response to the combined effects of growth temperature (a difference of ~50°C in their native environment) (Zinder et al. 1985; Franzmann et al. 1992) and the intracellular environment.

Materials and methods

Thermal denaturation studies and activation energy of unfolding

Irreversible thermal unfolding of EF-2 proteins was studied using differential scanning calorimetry (DSC) at three different scan rates (Thomas and Cavicchioli 2000; Thomas et al. 2001). The first-order rate constants for thermal unfolding (k_d) of EF-2 proteins were determined from the DSC scans as described by Sanchez-Ruiz et al. (1988). The logarithms of first-order rate constants for thermal unfolding (k_d) of all EF-2 proteins were plotted versus reciprocal temperature according to Arrhenius to calculate the activation energy of unfolding (E_a) (Stearn 1949).

The thermodynamic data for the transition state were calculated by the rearranged Eyring Absolute Rate Equation derived from the Transition State Theory (Eyring and Stearn 1939; Siddiqui et al. 1999, 2000) with the following equations:

$$\Delta H^* \left(\text{enthalpy of activation}\right) = E_a - RT \quad (1)$$

$$\Delta G^* \left(\text{free energy of activation}\right) = -RT\ln\left(\left(k_d h\right)/\left(K_B T\right)\right) \quad (2)$$

$$\Delta S^* \left(\text{entropy of activation}\right) = \left(\Delta H^* - \Delta G^*\right)/T \quad (3)$$

where T is the temperature, h is the Planck Constant (6.63×10^{-34} J s), K_B is the Boltzman Constant (1.38×10^{-23} J K^{-1}), and R is the Universal Gas Constant (8.314 J K^{-1} mol^{-1}).

The half-life ($t_{1/2}$) was calculated as $\ln 2/k_d$, where k_d is the first-order rate constant of thermal unfolding.

Temperature optimum and activation energy of ribosome-dependent GTP hydrolysis

Ribosome-dependent GTPase activity of EF-2 proteins was determined at various temperatures as described previously (Thomas and Cavicchioli 2000; Thomas et al. 2001). The activation energies (E_a) for GTP hydrolysis were determined by plotting the logarithm of GTPase activity of all EF-2 proteins versus reciprocal temperature according to Arrhenius (Stearn 1949). The thermodynamic data for the GTPase activity were calculated using Eq. 2, with the replacement of k_d with k_{cat}. The enthalpy and entropy of GTP hydrolysis were calculated using Eqs. 1 and 3, respectively.

The ratio (Q_{10}) of rate of GTPase activity at temperature T (= T_1) and T + 10 K (= T_2) was calculated as follows:

$$Q_{10} = \ln k_{T1}/k_{T2} = -\left(E_a/R\right) \times \left\{\left(T_2 - T_1\right)/\left(T_2 T_1\right)\right\} \quad (4)$$

where k_{T1} is the rate of reaction at T_1 and k_{T2} is the rate of reaction at T_2.

Results and discussion

Transition state of *Methanococcoides burtonii* and *Methanosarcina thermophila* EF-2

The calculation of the thermodynamic stability of proteins is based on the assumption that there is a rapid, reversible unfolding and refolding according to a simple two-state mechanism between the native, folded state (F) and the unfolded state (U). The stability of the protein is therefore defined as the difference in the free energy between F and U (Nosoh and Sekiguchi 1990). In contrast, the kinetic stability can be calculated for proteins following an irreversible

unfolding process where the native protein (F) changes reversibly to a partially unfolded transition state (TS*) before unfolding irreversibly (U). In this case, the magnitude of the free energy (ΔG*) difference between the folded (F) and the transition state (TS*) can be determined by the first-order rate constant k_d of the irreversible denaturation process (Violet and Meunier 1989). The irreversible, thermal denaturation of the protein results in the breakage of noncovalent linkages accompanied by an increase in the enthalpy of activation (ΔH*) and an opening of the protein structure with an increase in the disorder, or entropy, of activation (ΔS*).

Using DSC, the thermally induced unfolding (denaturation) of EF-2 from *Methanococcoides burtonii* and *Methanosarcina thermophila* was found to be irreversible (Thomas and Cavicchioli 2000; Thomas et al. 2001). From these experiments, the rate constant of unfolding was calculated, Arrhenius plots were constructed (Fig. 1), and the thermodynamic parameters for the activation of the transition state were determined (Table 1) over a broad temperature range of thermally induced denaturation.

The free energy of activation, ΔG*, for the *Methanosarcina thermophila* EF-2 was consistently higher than for the *Methanococcoides burtonii* EF-2 by as much as 13 kJ mol^{-1} when compared at the same temperatures (see Table 1), which indicates a more stabilized structure and results in a substantially increased half-life for the *Methanosarcina thermophila* protein. Even greater differences between the two proteins were observed when the parameters of enthalpy (ΔH*) and entropy (ΔS*) were compared. The *Methanococcoides burtonii* EF-2 exhibited less than 60% of the enthalpy value and less than 50% of the entropy of the *Methanosarcina thermophila* EF-2. For *Methanococcoides burtonii* EF-2, the reduced ΔH^* implies that fewer or weaker noncovalent bonds need to be broken to reach the transition state when compared with *Methanosarcina thermophila* EF-2 (see Table 1). In its transition state, the protein may be partially unfolded, and previously buried hydrophobic residues may become more exposed to the solvent in the *Methanococcoides burtonii* EF-2 compared to similar regions in the *Methanosarcina thermophila* EF-2. The exposed hydrophobic residues may cause an ordering of water molecules around them, thereby reducing the entropy of the transition state. It is noteworthy that both enthalpy and entropy changed concurrently (see Table 1). The apparent balance between changes in enthalpy and entropy has previously been described as the entropy–enthalpy compensation (Liu et al. 2000).

In comparison with *Methanococcoides burtonii*, at higher temperatures the *Methanosarcina thermophila* EF-2 has an increased enthalpy value (more or stronger noncovalent bonds), and this may prevent the protein from collapsing into its denatured form. As a consequence of the entropy–enthalpy compensation, the entropy of the transition state is also relatively high. As a strategy for thermal adaptation, it is interesting that the greater disorder of the *M. thermophila* EF-2 transition state would imply a greater overall flexibility of the protein. However, it must be noted that the transition state of denaturation is described here, not the ground state of the protein. In a contrasting study, the higher thermostability of bovine glutamate dehydrogenase compared with the enzyme from the Antarctic fish *Chaenocephalus aceratus* was attributed to entropic rather than enthalpic differences (Ciardiello et al. 2000). There is a lack of data regarding the activation parameters of denaturation of psychrophilic proteins, and additional studies are required on a range of proteins to determine whether entropy or enthalpy tends to control the unfolding of psychrophilic proteins.

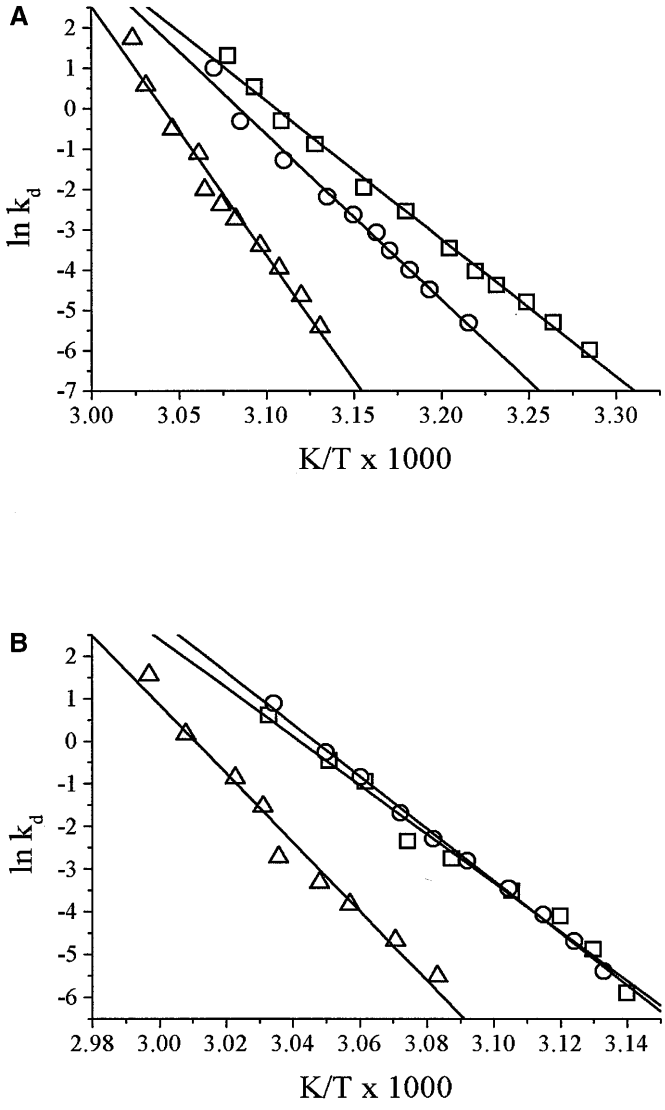

Fig. 1. Arrhenius plots for the determination of the activation energy (E_a) for the denaturation of EF-2 proteins from *Methanococcoides burtonii* (**A**) and *Methanosarcina thermophila* (**B**) in the absence of solute (*squares*), in the presence of 100 mM potassium aspartate (K-asp) (*circles*), and in the presence of 500 mM potassium glutamate (K-glu) (*triangles*). Activation energy (E_a) = slope × R; *K*, Kelvin; *T*, temperature

Effect of solutes on the transition state and unfolding

Methanosarcina thermophila accumulates up to 500 mM potassium glutamate (K-glu) in its cytoplasm in response to

Table 1. Thermodynamic activation parameters of irreversible denaturation of EF-2 proteins of *Methanococcoides burtonii* and *Methanosarcina thermophila* in the absence or presence of 100 mM potassium aspartate (K-asp) or 500 mM potassium glutamate (K-glu)

Solutes	*Methanococcoides burtonii* EF-2					*Methanosarcina thermophila* EF-2				
	T (°C)	ΔG* (kJ mol^{-1})	ΔH* (kJ mol^{-1})	ΔS* (J mol^{-1} K^{-1})	$t_{1/2}$ (min)	T (°C)	ΔG* (kJ mol^{-1})	ΔH* (kJ mol^{-1})	ΔS* (J mol^{-1} K^{1})	$t_{1/2}$ (min)
None	31.3	100.1	281.9	597.1	278	45.4	104.7	473.9	1,159.5	256
	33.3	99.1	281.8	596.8	141	46.4	102.3	473.9	1,163.1	92
	34.7	98.2	281.8	596.7	84	47.4	100.6	473.9	1,164.9	42
	36.3	97.7	281.8	595.3	55	48.9	99.5	473.9	1,162.8	23
	37.5	97.2	281.8	594.6	39	50.8	98.0	473.9	1,160.6	11
	39.0	96.2	281.8	595.1	22	52.2	97.3	473.9	1,157.7	7.0
	41.4	94.6	281.8	595.6	9.0	53.5	93.9	473.9	1,163.2	2.0
	43.8	93.7	281.8	593.7	5.0	54.7	93.0	473.9	1,162.1	1.0
	46.6	91.7	281.8	594.5	1.7	56.6	90.6	473.8	1,162.4	0.4
	48.6	90.8	281.8	593.7	1.0					
	50.2	89.0	281.8	596.3	0.4					
	51.8	87.3	281.8	598.4	0.2					
K-asp	37.9	100.9	337.1	759.5	141	46.1	103.5	506.5	1,262.4	153
	40.0	99.2	337.1	759.9	61	47.0	102.0	506.5	1,262.4	75
	41.2	98.3	337.1	760.0	38	48.0	100.2	506.5	1,264.1	41
	42.3	97.3	337.1	760.1	23	49.0	99.3	506.4	1,264.0	22
	43.1	96.4	337.1	761.2	15	50.3	98.0	506.4	1,263.0	11
	44.4	95.7	337.1	760.4	10	51.4	97.0	506.4	1,262.0	7.0
	45.9	94.9	337.0	759.0	6	52.4	95.6	506.4	1,262.1	4.0
	48.4	93.3	337.0	758.0	2.5	53.7	93.7	506.4	1,263.1	1.5
	51.0	91.5	337.0	757.5	1.0	54.8	92.4	506.4	1,262.7	1.0
	52.6	88.4	337.0	763.1	0.3	56.5	89.8	506.4	1,264.0	0.3
K-glu	46.3	103.7	510.2	1,272.7	156	51.2	105.6	669.0	1,737.1	172
	47.4	102.0	510.2	1,273.5	72	52.6	103.8	669.0	1,735.6	74
	48.7	100.6	510.2	1,272.7	36	54.0	101.9	669.0	1,733.5	32
	49.9	99.4	510.1	1,271.8	21	55.0	100.8	669.0	1,731.6	19
	51.3	98.1	510.1	1,270.0	11	56.3	99.6	669.0	1,728.3	11
	52.2	97.4	510.1	1,268.6	8.0	56.8	96.3	669.0	1,735.7	3.0
	53.2	96.7	510.1	1,266.8	5.0	57.7	95.0	669.0	1,735.0	1.5
	53.6	94.4	510.1	1,272.5	2.0	59.4	92.6	669.0	1,733.5	0.5
	55.2	93.2	510.1	1,269.8	1.0	60.6	89.1	669.0	1,737.6	0.1
	56.8	90.8	510.1	1,271.0	0.4					
	57.7	81.5	510.1	1,296.0	0.1					

The activation energies, E_a, were calculated form Arrhenius plots (Fig. 1)

increasing growth temperature (Thomas et al. 2001). The K-glu was shown to have a stabilizing effect on the EF-2 proteins from *Methanosarcina thermophila* and *Methanococcoides burtonii* (Thomas et al. 2001). Examining the thermodynamic parameters of transition state activation (see Table 1) indicates that the stabilizing effect of K-glu is manifested through an increase in the free energy ΔG* in association with increases in both ΔH* and ΔS*. The increased transition enthalpy indicates that K-glu aids the formation of noncovalent bonds in the native structure, or indirectly affects the surface tension of the solvent, thereby perturbing the stability of the protein.

Potassium aspartate (K-asp) is present (100 mM) in the cytoplasm of *M. burtonii* but was not detected in *Methanosarcina thermophila* (Thomas et al. 2001). A physiological level of 100 mM K-asp has less effect on the thermodynamic parameters of the transition state than does 500 mM K-glu (see Table 1), which indicates that the presence of K-asp in the cell is unlikely to be related to stabilizing proteins.

Nevertheless, the similar trends caused by K-asp and K-glu on ΔG*, ΔH*, and ΔS* (i.e., in the presence of solute all values increase) suggests that the nature of the interactions between protein and solute are similar for EF-2 proteins from both organisms. The mechanism of stabilization is not known; however, because of their zwitterionic character, glutamate and aspartate might form noncovalent bridges on the protein surface, or potassium might neutralize otherwise repulsive charges of amino acid side chains. Alternatively, they may exert an indirect effect on protein stability through the differential exclusion of water molecules and the subsequent effect on surface tension of the solvent (Timasheff 1998). The differing abilities of K-glu and K-asp to stabilize the EF-2 proteins may also reflect the concentrations examined (i.e., only physiologically relevant levels were studied) or their different chemical properties (i.e., length of side chain and hydrophobicity).

To further examine whether the solutes affect the mechanism of unfolding in a similar way for both proteins, the temperature of compensation (T_c) was calculated by plot-

ting ΔS* versus ΔH* (Barnes et al. 1969). A linear regression with a correlation coefficient (r^2) of 1.00 was obtained and a T_c value of 67°C was calculated. The linearity of the relationship indicates that, under the experimental conditions tested, the EF-2 proteins have the same mechanism of unfolding in the absence and presence of solutes.

The thermodynamic activation parameters (see Table 1) were also used to calculate thermodynamic stabilities at 0°C (ΔG_0*). The ΔG* values of the transition states for both proteins in the absence and presence of solutes were plotted against temperature (data not shown). Linear regressions were obtained ($r^2 > 0.985$) and the free energy was extrapolated to 0°C. The values without solutes, 100 mM K-asp and 500 mM K-glu, for *Methanococcoides burtonii* EF-2 were 119, 130, and 158 kJ mol^{-1}, respectively, and for *Methanosarcina thermophila* EF-2 they were 157, 162, and 195 kJ mol^{-1}, respectively. This result clearly shows that the *M. thermophila* EF-2 in the presence of 500 mM K-glu is more stable and *Methanococcoides burtonii* EF-2 in the absence of solutes is less stable.

Thermodynamics of GTPase activity and its implication for thermal adaptation

The *Methanococcoides burtonii* EF-2 has a low activation energy of GTP hydrolysis (Thomas and Cavicchioli 2000) and a high k_{cat}/K_m ratio at low temperature (Thomas et al. 2001), characteristics that have been described as typical of cold-active enzymes (Feller and Gerday 1997; Gerday et al. 1997). Arrhenius plots were constructed (Fig. 2), and thermodynamic analyses of enzyme catalysis show that the ΔG* values are similar for the *Methanococcoides burtonii* and *Methanosarcina thermophila* proteins over the temperature range examined (Table 2) and are similar to ΔG* for the EF-2 protein from the thermoacidophilic archaeon *Sulfolobus solfataricus* at 87°C (ΔG* = 83 kJ mol^{-1}) (Raimo et al. 1995). However, the contribution of the reaction enthalpy was different for these three proteins. The *Methanococcoides burtonii* EF-2 has a lower ΔH* value (75 kJ mol^{-1}) at 40°C compared with *Methanosarcina thermophila* EF-2 (88 kJ mol^{-1}). The value for ΔH* was also higher for the *Sulfolobus solfataricus* EF-2 (ΔH* = 82 kJ mol^{-1} at 87°C) (Raimo et al. 1995) compared to *Methanococcoides burtonii* EF-2.

It has been speculated that a reduced reaction enthalpy may give an advantage for enzymatic activity at low temperature by reducing the temperature dependence of k_{cat} (Lonhienne et al. 2000). It was demonstrated that the difference between enthalpies for psychrophilic and mesophilic enzymes from bacteria and eukaryotes Δ(ΔH*) was between 4.5 and 42 kJ mol^{-1} whereas the differences between the free energies Δ(ΔG*) did not exceed 4.1 kJ mol^{-1} (Lonhienne et al. 2000). Consistent with these

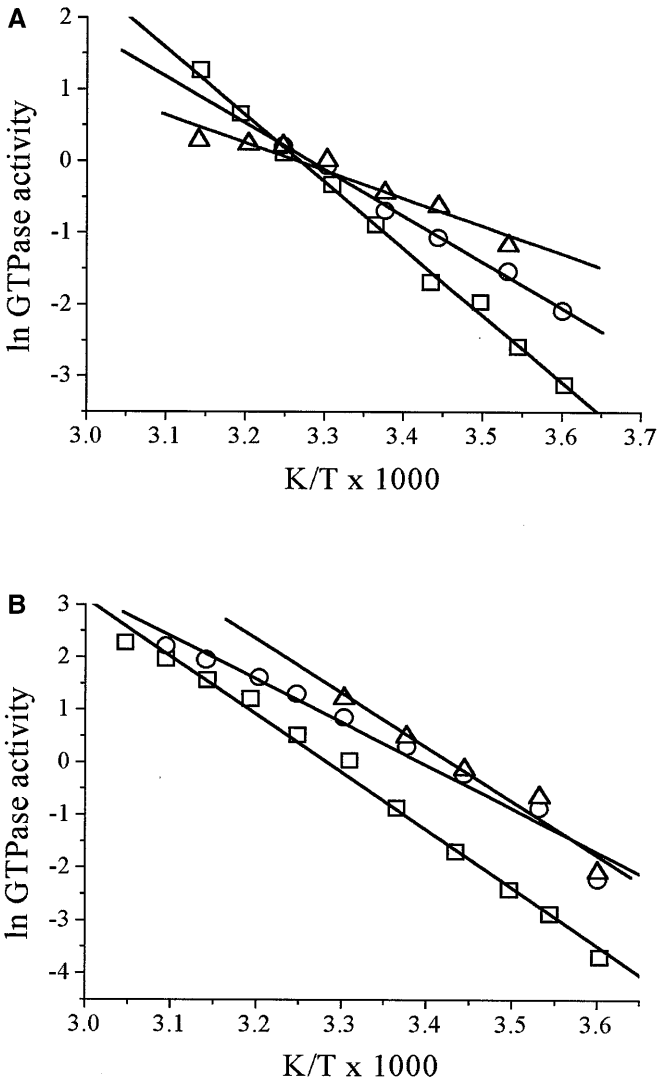

Fig. 2. Arrhenius plots for the determination of activation energy (E_a) for guanosine triphosphate (GTP) hydrolysis by EF-2 proteins from *Methanococcoides burtonii* (**A**) and *Methanosarcina thermophila* (**B**) in the absence of solute (*squares*), in the presence of 100 mM K-asp (*circles*), and in the presence of 500 mM K-glu (*triangles*). Activation energy (E_a) = slope × R; *K*, Kelvin; *T*, temperature

Table 2. Thermodynamic parameters for ribosome-dependent GTPase activity of *Methanococcoides burtonii* and *Methanosarcina thermophila* at various temperatures

Parameter	*Methanococcoides burtonii*					*Methanosarcina thermophila*				
	10°C	23°C	30°C	40°C	50°C	10°C	23°C	30°C	40°C	50°C
ΔH* (kJ mol^{-1})	76.0	75.9	75.9	75.0	75.7	88.7	88.5	88.5	88.0	88.3
ΔG* (kJ mol^{-1})	80	84	85	86	87	80	83	85	84	84
ΔS* (kJ mol^{-1} K^{-1})	–15	–26	–31	–34	–36	31	18	11	14	12

findings, the Δ(ΔH*) and Δ(ΔG*) values between the *Methanococcoides burtonii* and *Methanosarcina thermophila* EF-2 proteins were approximately 13 and less than 3 kJ mol^{-1}, respectively.

As a consequence of the different enthalpy values, the entropies for *Methanococcoides burtonii* and *Methanosarcina thermophila* EF-2 proteins have opposite signs (see Table 2). The positive entropy values for the *Methanosarcina thermophila* EF-2 imply that the activated transition state of catalysis is more disordered than the ground state whereas the negative values for *Methanococcoides burtonii* indicate increased order. This observation, however, may not relate solely to the direct enzyme–substrate interactions because the formation of the transition state involves not only complex interactions of the protein with the substrate but also interactions with the solvent. In *Thermus thermophilus* EF-G (the bacterial homologue of EF-2), the binding of guanosine diphosphate (GDP) (and probably GTP) has been shown to involve numerous interactions of the nucleotide with water molecules and magnesium ions (Al-Karadaghi et al. 1996; Chirgadze 1996). The formation of hydrogen bonds in the enzyme–substrate complex also involves the displacement of hydrogen-bonded water molecules, which results in an increase in entropy. Entropy gains of about 40 J K^{-1} have been calculated per mole of water molecule released (Fersht 1984). Thus, the Δ(ΔS*) of *Methanococcoides burtonii* and of *Methanosarcina thermophila* EF-2 of 48 J K^{-1} (Table 3) could indicate that one extra water molecule is displaced during catalysis in *Methanosarcina thermophila* EF-2.

The relative differences of entropy [Δ(ΔS*)] indicate in general that the activated state of the *Methanococcoides burtonii* EF-2 undergoes more ordering than does the *Methanosarcina thermophila* EF-2 (see Table 2). As discussed by Lonhienne et al. (2000), the decreased number of enthalpy-driven interactions that need to be broken during activation in cold-active enzymes could lead to the catalytic region of the protein having a more flexible structure. As a consequence, the ground state of the enzyme–substrate complex may occupy more conformational states, which subsequently undergo ordering during activation and hence lead to a greater decrease in entropy for a psychrophilic protein when compared to its mesophilic or thermophilic counterparts. Similar trends were reported recently in a comparative study examining the formation of the activated state for two psychrophilic chitinases and one mesophilic bacterial chitinase (Lonhienne et al. 2001). Although the reaction enthalpy was reduced and the entropy values were negative for the cold-adapted enzymes, the mesophilic enzyme showed positive entropy, indicating a more disordered activation state. It is possible that the low ΔS* of cold-active enzymes, and hence the higher flexibility of regions of the protein, reflects a reduced ability to displace water molecules in (or around) the active site. In the same way, a more rigid structure of a mesophilic or thermophilic protein may displace more water molecules when the activated substrate binds to the active site.

The free energy barrier (ΔG*) for *Methanococcoides burtonii* EF-2 increased by 7 kJ mol^{-1} from 10° to 50°C compared to only 4 kJ mol^{-1} for the EF-2 from *Methanosarcina thermophila* (see Table 2). This increase in ΔG* with temperature probably reflects conformational changes within the catalytic cleft of both proteins that may demand higher energy contributions to form the transition state. Structural data for the *Methanococcoides burtonii* EF-2 are limited to homology modeling with the EF-G protein from the bacterium *Thermus thermophilus* (Thomas and Cavicchioli 1998). This limitation restricts our ability to interpret the thermodynamic data in a structural context. Nevertheless, it seems plausible that the higher ΔG* value for *Methanococcoides burtonii* EF-2 may be a result of temperature-induced, conformational changes that are greater than in *Methanosarcina thermophila* EF-2 and which might be a consequence of the expected higher flexibility of the psychrophilic enzyme.

Effect of solutes on the thermodynamics of activity

Physiological levels of solutes have a pronounced effect on EF-2 stability, the reaction rate, the Michaelis–Menten constant, and the activation energy for GTPase activity (Thomas et al. 2001; see Table 3). Thermodynamic analysis revealed that the free energy of activation is similar in the absence or presence of solutes (Table 3). However, in all cases the addition of solutes lowered the reaction enthalpy, indicating that they facilitate the breaking of enthalpy-driven interactions during catalysis. This reaction might

Table 3. Thermodynamic parameters for GTPase activity of *Methanococcoides burtonii* and *Methanosarcina thermophila* at 40°C under different conditions

	Methanococcoides burtonii			*Methanosarcina thermophila*		
	None	K-asp (100 mM)	K-glu (500 mM)	None	K-asp (100 mM)	K-glu (500 mM)
k_{cat} (min^{-1})	2.0	1.2	1.3	3.7	5.3	2.3
K_m (μM)	21	13	9	33	33	12
E_a (kJ mol^{-1})	78	54	32	91	68	85
ΔH* (kJ mol^{-1})	75	52	29	88	65	82
ΔG* (kJ mol^{-1})	86	87	87	84	83	85
ΔS* (kJ mol^{-1} K^{-1})	–35	–112	–185	13	–57	–10
Q_{10}	2.7	2.0	1.5	3.2	2.4	2.6

The activation energies, E_a, were calculated form Arrhenius plots (Fig. 2)

occur either through their direct interaction with the catalytic site or through an alteration of the interaction of EF-2 with the ribosome. The activity of the EF-2 proteins is strongly dependent on the presence of ribosomes (Thomas et al. 2001), and recent structural studies on the bacterial system have shown complex interactions and molecular cross-talking between EF-G and the ribosome (Frank et al. 1995; Agrawal et al. 1998, 1999; Rodnina et al. 1999; Frank and Agrawal 2000). The changes in entropy observed between solute and nonsolute conditions (see Table 3) may also indicate that the solvent (ions or water) has altered mobility in the presence of K-asp and K-glu. This idea is supported by the entropy values (Table 3) for the release of water molecules, which indicate that several water molecules could potentially be differentially displaced between solute and nonsolute conditions. It will be interesting to examine this hypothesis when structural data for the EF-2 proteins become available.

The effects of solutes on the thermodynamic properties of EF-2 activity demonstrate that the production of 100 mM K-asp by *Methanococcoides burtonii* would be beneficial for EF-2 activity in vivo by reducing the temperature dependence of activity (Q_{10} = 2.0 versus 2.7 in its absence). Interestingly, 500 mM K-glu may produce an even greater benefit for low-temperature activity, although the lack of such high-level accumulation may indicate that other cellular functions could be compromised.

To further examine the effects of solutes on the thermodynamic properties of activity and stability, a comparison was made between the ΔG_0^* values of stability (in the absence of substrate or ribosomes) and T_{opt} values (in the presence of substrate and ribosomes) (Thomas et al. 2001) for both proteins in the presence or absence of solutes. For *Methanosarcina thermophila* EF-2, the highest thermostability occurred in the presence of K-glu whereas T_{opt} values were lowest. In contrast, the least thermostable protein was the *Methanococcoides burtonii* EF-2 in the absence of solute; however, T_{opt} was high (Thomas et al. 2001). These data indicate that, although K-glu and ribosomes stabilize EF-2 proteins, temperature affects the activity of the two proteins differently in the presence of K-glu. Although the mechanisms explaining these differences are not clear, these findings highlight the importance of examining the effects of thermodynamic parameters on both unfolding and catalysis.

The calculation of thermodynamic parameters for protein stability and activity over a range of physiological temperatures has provided new insight into how the EF-2 proteins from *Methanococcoides burtonii* and *Methanosarcina thermophila* respond to temperature changes, and hence the evolutionary strategies that have been adopted by these microorganisms for different thermal environments. This article is the first report of thermodynamic parameters for low-temperature-adapted proteins from Archaea, and the findings are consistent with recent studies on bacterial and eukaryotic proteins (Ciardiello et al. 2000; Lonhienne et al. 2000, 2001). Taken together, these studies demonstrate that although general principles of low-temperature adaptation of cold-active proteins may be proposed (e.g., higher activity at low temperature), the nature of individual proteins and their specific intracellular or extracellular context are likely to have a major effect on the cellular thermal adaptation strategies that have evolved.

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