Analysis of Thermal Stabilizing Interactions in Mesophilic and Thermophilic Adenylate Kinases from the Genus Methanococcus*

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Adenylate kinases (ADKs) from four closely related methanogenic members of the Archaea (the mesophilic Methanococcus voltae (MVO), the thermophile Methanococcus thermolithotrophicus (MTH), and the extreme thermophiles Methanococcus igneus (MIG) and Methanococcus jannaschii (MJA)) were characterized for their resistance to thermal denaturation. Despite possessing between 68 and 81% sequence identity, the methanococcal ADKs significantly differed in their stability against thermal denaturation, with melting points ranging from 69 to 103 °C. The high sequence identity between these organisms allowed regions of the MVO and MJA ADKs to be exchanged, producing chimeric ADKs with significantly altered thermal stability. Up to a 20 °C increase or decrease in stability was achieved for chimeric ADKs, whereas 88% of the original protein sequence was maintained. Based on our previous structural modeling studies, we conclude that cooperative interactions within the hydrophobic protein core play an integral role in determining the differences in structural stability observed between the methanococcal ADKs. From comparisons of the effects of temperature on protein unfolding and optimal enzymatic activity, we also conclude that thermostability and enzymatic temperature optima are influenced differently by molecular modifications and thus that the protein flexibility required for activity and stability, respectively, is not unconditionally linked within the methanococcal ADKs.

There has recently been a drastic increase in the isolation and investigation of microorganisms living under conditions of extreme temperature, pH, or salinity (1). Of particular interest have been studies to delineate those strategies by which hyperthermophiles grow and flourish in natural environments that approach and even exceed 100 °C. This research has focused mainly on the enzymes isolated from these hyperthermophilic organisms and their intrinsic ability to function and remain stable at or near the optimal growth temperature of the organism (2–5).

Although our understanding of protein stability is currently limited, it is apparent that enzymes use different combinations of subtle intramolecular interactions for functional adaptation to high temperatures. These interactions may include hydrogen bonding (3, 6, 7), ionic interactions (8–12), the burying of hydrophobic residues (13–17), minimization of covalent modifications (18, 19), the stabilization of helices (20–23), reduced chain flexibility (24–27), and the stability of oligomeric interactions (28, 29) (for reviews, see Refs. 7, 30, and 31).

Despite numerous comparative studies of proteins from mesophilic and thermophilic organisms, the structural strategies employed in proteins to function in a particular thermal environment have not been fully identified. The interpretation of comparative studies has been limited for several reasons. On the one hand, the net free energy of stabilization of proteins is quite small, and minor changes in structure can significantly affect stability (32, 33); yet numerous changes can be introduced into most proteins with little effect on overall structure or stability (34–36). Thus, thermoadaptive features are often masked by a background of evolutionary sequence divergence, making interpretation of structural differences difficult. Clearly, comparing sets of enzymes from organisms that are phylogenetically related best minimizes the uncertainty in this kind of approach.

Members of the archaeal genus Methanococcus offer an opportunity to compare thermophilic and mesophilic enzyme homologs that share substantial sequence identity (2). We have previously isolated, sequenced, and modeled the structures of the adenylate kinases (ADKs)1 from the mesophilic Methanococcus voltae (MVO), the moderately thermophilic Methanococcus thermolithotrophicus (MTH), and the extremely thermophilic Methanococcus igneus (MIG) and Methanococcus jannaschii (MJA) (37–39). These enzymes share between 68 and 81% sequence identity, yet their temperature optima for enzymatic activity are 37, 68, 80, and 83 °C respectively.

In this study, the wild-type methanococcal ADKs and a series of chimeric ADKs were expressed in Escherichia coli and subsequently analyzed for structural stability versus thermal inactivation and denaturation. Our results lead to two major conclusions: 1) the composition of the terminal regions of the methanococcal ADKs makes a major contribution to their thermal stability and optimal temperature for activity; and 2) the temperature for optimal activity and the thermal stability of the methanococcal ADKs are influenced differently by sequence modifications.

EXPERIMENTAL PROCEDURES

Generation of Chimeric Enzymes—The methanococcal ADKs contain substantial regions of sequence homology (see Fig. 2). This allowed the generation of hybrid enzymes using the polymerase chain reaction to amplify and then link regions of the MVO and MJA adkA genes (see Olsen et al. (40) and Yon and Fried (41) for a description of this method). The methanococcal adkA genes, cloned into the pET11b vector (Novagen), were used as the target DNA for all polymerase chain reaction amplifications. Amplifications were performed using Vent polymerase...
and primers homologous to the lac operator, T7 terminator, and conserved regions between MVO and MJAdk genes (nucleotides 100–119 and 457–477). Site-specific mutagenesis was subsequently performed using mismatched primers in polymerase chain reactions. Polymerase chain reaction fragments coding for chimeric enzymes were cloned into pET11b for expression in E. coli. Each protein-coding region was subsequently sequenced in both directions.

Overexpression and Purification of Chimeric and Wild-type ADKs—E. coli cells (BL21(DE3)) harboring plasmids directing synthesis of wild-type and chimeric ADKs were grown to an absorbance of 0.6 at 600 nm in the presence of 100 mg/liter ampicillin and then induced with 1 mM isopropyl-β-D-thiogalactopyranoside. The cultures were incubated for an additional 2–3 h before harvesting by centrifugation. Cells were resuspended and then ruptured by passage through a French press before clarification by centrifugation. Cell extracts containing thermophilic ADKs were heated at 70 °C for 30 min to denature the E. coli proteins, which were then removed by centrifugation at 303,000 × g for 1 h. ADKs were purified from the supernatant by affinity chromatography on a Cibacron blue column (Sigma) as described previously (37).

Enzyme Assay—All assays are performed at a final ADK concentration of ~0.0001 mg/ml. For each experiment, ADK was added to a mixture containing 1.0 mM glucose, 0.4 mM NADP⁺, 100 mM KCl, 2 mM MgCl₂, and 50 mM Tris-HCl (pH 7.7). Assay solutions were preheated at the desired temperature for at least 4 min before the reaction started by the addition of ADP (2.5 mM final concentration). Assay solutions were incubated for 3 min before stopping the reactions. For thermal stable ADKs such as MJA, J160V, V36J, VJV, and JVJ, reactions were stopped by rapid cooling in an ice bath. No appreciable activity was observed for these ADKs below 20 °C. Reactions containing mesophilic ADKs were stopped by the addition of perchloric acid (6% final concentration), followed by neutralization and precipitation with KOH and KHCO₃ (0.09 and 0.41 x final concentrations, respectively). The thermophilic methanococcal ADKs could not be acid-denatured. Final ATP levels were measured using an ATP-dependent reduction of NADP⁺ to NADPH involving hexokinase and glucose-6-phosphate dehydrogenase. This reaction was allowed to proceed to completion at 4 °C (-5 min) and then analyzed for absorbance changes at 340 nm. Assays were repeated several times.

Circular Dichroism—The CD spectra were measured on a Jasco J-720 spectrophotometer (Japan Spectroscopic Co., Ltd.) at protein concentrations of 0.25–0.35 mg/ml in 10 mM phosphate buffer (pH 8.0) and an optical path length of 1 mm.

Thermal Denaturation—Thermal denaturation curves were obtained by monitoring changes in ellipticity at 222 nm as temperature was increased at a rate of 1.5–3 °C/min. All proteins examined in this study denatured irreversibly at elevated temperatures, preventing direct determination of Tm. Therefore, the midpoint of rapid denaturation was used to estimate the melting point (Tm) of 0.5 °C. Long exposure of the ADKs to temperatures below that where rapid denaturation occurred resulted in gradual denaturation and aggregation of the protein. Fast heating rates and high starting temperatures were therefore required to estimate the denaturation midpoints. The spectrum of completely denatured ADK lacked any significant absorption between 190 and 250 nm.

Guanylinium Cl Denaturation—For chemical denaturation, each protein (0.3 mg/ml) was equilibrated at 25 °C in phosphate buffer containing varying concentrations of GdmCl for a minimum of 40 min prior to measurements. Protein denaturation was monitored by changes in CD measurements at 222 nm. Each protein reversibly unfolded in the presence of GdmCl in a single cooperative manner. For the extremely thermophilic MJAdk, V36J, and J160V ADKs, thermal denaturation incorporating varying GdmCl concentrations was performed under conditions identical to those described above. Despite the presence of GdmCl, thermal denaturation remained largely irreversible.

RESULTS

Construction of Chimeric ADKs—We utilized a set of chimeric adenylate kinase enzymes that combine structural regions of the mesophilic M. voltae (MVO) and hyperthermophilic M. jannaschii (MJA) ADKs to identify structural features that determine the overall thermal stability and temperature activity optimum. The high degree of sequence identity and similarity between the MVO (designated V) and MJA (designated J) ADKs facilitated the generation of chimeric ADKs. For this study, the 36-residue amino-terminal region or the 32-residue carboxyl-terminal region was exchanged to produce chimeras J36V, V36J, V160J, and J160V. Thus, J36V differs from MVO only by the 13 nonconserved residues donated by the MJA N-terminal fragment. Similarly, V36J differs from MJA only by the 13 nonconserved residues donated by the MVO N-terminal fragment. Chimeras formed by the exchange of C-terminal fragments differed from the native enzyme by the 11 nonconserved residues that accompanied fragment exchange. Chimeras with both terminal regions exchanged were also constructed (JVJ and VJV) (Figs. 1 and 2). Overall, chimeras JVJ and VJV retain 88% sequence identity to the recipient ADK and have 80% identity to the ADK that donated the two ends. These exchanges resulted in the creation of fully functional chimeric enzymes with dramatically altered thermal properties (see below). To characterize the stabilizing interactions within specific regions of the protein, mutations were also introduced into the chimeric proteins JVJ and VJV. These mutations were introduced in pairs so as to increase the chances of identifying potentially small changes in thermostability (see Figs. 1 and 2 for a detailed description of the constructs). Analysis of the circular dichroism spectrum between 190 and 300 nm for each of the chimeras indicated that the chimeric enzymes were identical to the MVO and MJAdk in overall secondary structure composition. The effects sequence differences between the MVO and MJAdk have on thermal stability and activity were assessed by examining the temperature optimum and temperature-dependent denaturation profile for each native and chimeric ADK.

Thermostability—Thermal denaturation midpoints were determined by monitoring changes in secondary structure content as monitored by CD analysis at 222 nm. As can be seen, native MVO and MTH ADKs denatured irreversibly with midpoints of 69 and 86 °C, respectively (Figs. 3 and 4). However, for the extremely thermophilic MIG and MJAdk, rapid
unfolding was not achieved within the temperature range limitation of the assay, indicating melting midpoints in excess of 95 °C. To estimate the $T_m$ of the MJA ADK, we monitored denaturation in the presence of increasing amounts of GdmCl and estimated the $T_m$ at zero GdmCl by extrapolation (Fig. 3).

Using this method, the melting point of the MJA ADK was estimated to be between 102 and 105 °C, 34 °C higher than that of the MVO ADK. The validity of this approach was verified by our finding that it accurately predicts the experimentally determined melting point of the MTH ADK in the absence of denaturant. For chimeras V36J and J160V, the addition of GdmCl was also necessary to estimate a $T_m$ (Fig. 3).

The thermal denaturation profiles for the 13 chimeric ADKs are shown in Fig. 4. The addition of either terminal region of the MJA enzyme to the MVO enzyme led to chimeras (J36V and V160J) that manifested only a 4–5 °C increase in thermal stability compared with MVO (Fig. 4A). In contrast, chimera JVJ, which contained both N- and C-terminal regions donated by MJA, displayed a thermal stability that was 20 °C higher than MVO (Fig. 4A). A similar but opposite trend was seen for the replacement of the MJA termini with the corresponding regions of the mesophilic MVO ADK. Whereas the $T_m$ of VJV was 20 °C less than that of the highly thermophilic MJA, the $T_m$ values of V36J and J160V were only 5 and 7 °C lower than that of MJA (Figs. 3 and 4B). Additional changes in JVJ and VJV by the introduction of site-specific exchanges at several residue positions (see Fig. 1 for details) led to varying changes in thermal stability, ranging from a 1 °C increase in stability to a 10 °C decrease in stability (Figs. 1 and 4A and B).

Examination of the thermal denaturation profiles reveals complex transitions in which the major steep and, presumably, cooperative transition is most often preceded by a less steep and variable transition that occurs at lower temperature. Although it is possible that these results reflect a transition between two states and an even more complex set of transitions, the irreversible nature of ADK thermal denaturation does not allow a detailed and conclusive interpretation of the CD profiles.

**Temperature Optimum**—The exchange of the terminal regions between the MVO and MJA ADKs altered the optimal temperature of each enzyme in a direction that reflected the temperature optimum of the enzyme that donated the terminal region. The temperature optima of chimeras J36V, J160V, and JVJ were 8, 16, and 23 °C higher than that of the MVO enzyme (37 °C) (Fig. 5A). In comparison, chimeras V36J, J160V, and VJV had temperature optima that were 11, 8, and 13 °C, respectively, below that of the MJA enzyme (83 °C) (Fig. 5B).
altered thermal stability of JVJ and VJV by the introduction of several additional mutations (see Fig. 1) did not alter the temperature optima of the chimeras. Each of the chimeras displayed a specific activity similar to that of the native enzymes, with the exception of JVJ182,185, which had significantly reduced activity levels (data not shown).

**DISCUSSION**

Despite 68% amino acid sequence identity, the temperature optima and thermal melting points of the MVO and MJA ADKs were found to be separated by 45 and 34 °C, respectively. The production and examination of several chimeric enzymes containing regions of these mesophilic and thermophilic ADKs have identified a set of structural modifications responsible, in large part, for the mesophilic and thermophilic nature of the MVO and MJA ADKs.

Whereas the replacement of the 36-residue N-terminal region of MVO with the homologous region derived from MJA to form chimeras J36V and V36J or the replacement of the 32-residue C-terminal region of MVO with the homologous region derived from MJA to form V160J and J160J led to single-substitution chimeras JVJ and VJV further revealed the importance of the hydrophobic core in determining the overall protein stability of the methanococcal ADKs. J32,36VJ, which was generated from JVJ by the introduction of Val and Met at positions 32 and 36, respectively, exhibited a 6.5 °C decrease in temperature stability compared with JVJ (Val and Met reside at positions 32 and 36, respectively, in MVO). In contrast, the stability of V32,36JV, in which Val-32 and Met-36 were both replaced by introduction of Ile (present at positions 32 and 36 in MJA) was not significantly different from that of VJV.

The results of these changes can be understood by consideration of our previously modeled structures for the methanococcal ADKs (39). This study suggested that residue 32 interacts with residue 19 in the N-terminal region of the protein and residue 188 in the C-terminal region (Fig. 6). These correspond to the interaction of Ile-32 with Val-19 and Ile-188 in MJA and to the interaction of Val-32 with Ser-19 and Thr-188 in MVO. Additionally, residue 36 was predicted to partially interact with residues 20 and 24 (Ile with Thr and Ile in MJA; Met with Ser and Met in MVO). The stabilizing influence of these hydrophobic interactions would not be operative in MVO since MVO Ser-19 and Thr-188 would not participate in stabilizing hydrophobic interactions. Thus, the change from Ile-32 and Ile-36 present in JVJ to Val-32 and Met-36 in J32,36VJ would lead to a disruption in the hydrophobic interactions normally occurring in the MJA ADK and thus explain the reduced stability observed for J32,36VJ. In contrast, the substitution of Val-32 and Met-36 in VJV with Ile-32 and Ile-36 to create V32,36JV would not be expected to lead to a change in thermal stability since the hydrophobic associations are already absent.

The effects of amino acid exchanges introduced into chimera JV117,120J further demonstrated the sensitivity of ADK stability to core modifications. It might have been expected that the highly conservative Leu-to-Ile and Val-to-Leu changes introduced into JVJ to generate JV117,120J would not have had a significant effect on thermal stability, yet a dramatic 10 °C loss in stability was observed. In accordance with our structural model, we believe that the introduction of Ile-117 and Ile-120 to form JV117,120J in combination with the neighboring Ile-119 and Ile-173 significantly increased the side chain volume near the peptide backbone at the β-carbons, leading to thermal
destabilization due to suboptimal packing caused by steric interference. Although we fully appreciate the speculative nature of these interpretations, our results strongly suggest that cooperative interactions and packing within this core region are very important determinants for thermal stability.

Although our thermal denaturation studies on chimeric ADKs clearly demonstrate the importance of the amino- and carboxyl-terminal regions in determining the overall stability of the methanococcal ADKs, analysis of enzymatic temperature optima indicates that these structural features contribute to catalytic activity and overall protein stability differently. The properties of chimera V160J and the effects of site-specific mutagenesis on chimeras JVJ and VJV best exemplify a partial separation between interactions influencing stability and activity. Whereas construction of chimera V160J resulted in an ~16 °C increase in temperature optima (Fig. 5A), overall thermostability was only changed slightly (+5 °C) (Fig. 4A). Similarly, mutations to chimeras JVJ and VJV had a variety of effects on stability, but failed to have any influence on temperature optima (Fig. 1). These differing results suggest that the temperature optima for enzymatic activity of these ADKs are not determined by the overall flexibility/stability of the protein. This conclusion is consistent with previous studies that have suggested that the active-site region of the enzyme is more flexible than the molecule as a whole (42).

In the course of binding substrates, adenylate kinases undergo two large domain shifts: the first when the protein closes over bound AMP and the second when the large lid domain closes over the active site upon ATP binding in a proposed hinge-like movement between rigid bodies (43–46). Based on our previous modeling studies and assuming a similar substrate binding scheme for the methanococcal ADKs, the terminal regions that we exchanged to construct the MOV/MJA chimeras would not be directly involved in the large domain movements that occur during the substrate-binding cycle. However, they do contain regions that our modeling studies predict would be in close proximity to the proposed hinge sites (39). The results of this study suggest that the temperature optimum for activity of adenylate kinases may be more dependent on the mechanics of conformational movements and substrate binding than the protein’s overall flexibility/stability. This can be seen in the ~10 °C lower temperature optimum of JVJ compared with VJV (60 versus 70 °C, respectively) despite its greater thermal stability ($T_m$ = 85 versus 82.5 °C).

Although our studies demonstrate that the N- and C-terminal regions of the methanococcal ADKs play a major role in determining overall thermal stability and enzymatic activity, it is apparent that additional mutational studies and a complete structural analysis are required to delineate the full range of structural features that confer structural and functional integrity at elevated temperature. These detailed studies are currently underway.

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