Zinc, a Novel Structural Element Found in the Family of Bacterial Adenylate Kinases†,‡

Philippe Glaser,§ Elena Presecan,∥ Muriel Delepierre,⊥ Witold K. Surewicz,*,# Henry H. Mantsch,○ Octavian Bârzu,∥ and Anne-Marie Gilles*,∥

Unité de Régulation de l'Expression Génétique, Unité de Biochimie des Régulations Cellulaires, and Laboratoire de Résonance Magnétique Nucléaire, Institut Pasteur, Paris, France, and Institute of Biological Sciences, National Research Council, Ottawa K1A 0R6, Canada, and Institute of Biodiagnostics, National Research Council, Winnipeg R3B 1Y6, Canada

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ABSTRACT: The *adk* gene from *Bacillus stearothermophilus* was cloned and overexpressed in *Escherichia coli* under the control of the *lac* promoter. The primary structure of *B. stearothermophilus* adenylate kinase exhibited 76% identity with the enzyme from *Bacillus subtilis*, 60% identity with the enzyme from *Lactococcus lactis*, and 42% identity with the enzyme from *E. coli*. The most striking property of the adenylate kinase from *B. stearothermophilus* is the presence of a structural zinc atom bound to four cysteines in a zinc finger-like fashion. The ability to coordinate zinc is predicted also for a number of other isoforms of bacterial adenylate kinases. Furthermore, the tightly bound metal ion contributes to the high thermodynamic stability of adenylate kinase from *B. stearothermophilus*.

One important goal of protein chemistry is to elucidate the structural factors that are involved in the formation and maintenance of thermodynamically stable structures (Matthews, 1987; Nosoh & Sekiguchi, 1990). Our ability to study in a systematic way the rules governing protein stability has increased greatly with the advent of recombinant DNA technology and methods of site-directed mutagenesis (Nicholson et al., 1988; Kanaya et al., 1991). Additional clues guiding rational design of thermostable enzymes may be obtained from a comparison of homologous proteins from mesophilic and thermophilic organisms (Brock, 1985; Argos, 1989).

Adenylate kinase (AK, ATP–AMP phosphotransferase, EC 2.7.4.3)[1] is a small monomeric protein with relatively well conserved primary structure among several species (Schulz et al., 1986). It represents a suitable model for structural and thermodynamic analysis of protein stability. Our previous studies (Gilles et al., 1986; Rose et al., 1991a) have indicated that the four residue long sequence ^{85}GlyPheProArg^{88}, which forms a β-turn bracketed by a β-sheet and an α-helix, plays a crucial role in stabilizing the tertiary structure of the enzyme. Recently, we have focused our effort on understanding the structural and functional properties of adenylate kinases of enhanced stability.

In this report, we describe the molecular cloning of the *adk* gene from *Bacillus stearothermophilus* and characterization of the protein expressed in *Escherichia coli*. An unexpected property of this enzyme is the presence of a structural zinc atom bound to four cysteines. The presence of a tightly bound metal ion contributes to the strikingly high thermodynamic stability of adenylate kinase from *B. stearothermophilus*.

Experimental Procedures

Materials. Adenine nucleotides, restriction enzymes, T4 DNA ligase, and coupling enzymes were obtained from Boehringer Mannheim. The DNA polymerase large fragment (Klenow) was from Du Pont-New England Nuclear. TPCK-treated trypsin, soybean trypsin inhibitor, PMPS, and 4-(2-pyridylazo)resorcinol were purchased from Sigma, and blue-Sepharose was from Pharmacia LKB Biotechnologies Inc. Oligonucleotides were synthesized using the phosphoamidinate method on a commercial DNA synthesizer (Cyclone, Biosearch).

Bacterial Strains, Plasmids, Growth Conditions, and DNA Manipulations. The *B. stearothermophilus* strain was obtained from the Collection Nationale de Culture de Microorganismes, Institut Pasteur, Paris. The temperature-sensitive *E. coli* strain CR341 T28 (F$^-$, *thr, leu, ile, thy, lac*Y, *met*, and *adk*^{ts}) (Kohyiama et al., 1966) was used for the cloning of the *B. stearothermophilus adk* gene by complementation of the thermosensitive growth phenotype. The XL1 Blue *E. coli* strain (Bullock et al., 1987) was used for DNA sequencing. The cloned *adk* gene was overexpressed in the TG1 *E. coli* strain (Gibson, 1984) harboring plasmid pDIA17 which encodes the *lac*I gene (Munier et al., 1991). *B. stearothermophilus* chromosomal DNA was prepared as described for *Bacillus subtilis* (Saunders et al., 1984). *E. coli* strains were grown in LB medium (Miller, 1972) supplemented with 0.01% thymine, 100 μg/mL ampicillin, and 20 μg/mL chloramphenicol when required. To overproduce the AKst, the *lac* promoter was induced by 1 mM isopropyl β-D-thiogalactoside when the culture reached an absorbance of 0.5. Bacteria were harvested by centrifugation 4 h after induction.

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* Address correspondence to either author.

§ Unité de Régulation de l'Expression Génétique.

∥ *Unité de Biochimie des Régulations Cellulaires.*

⊥ Laboratoire de Résonance Magnétique Nucléaire.

Institute of Biological Sciences.

○ Institute of Biodiagnostics.

[1] Abbreviations: AK, adenylate kinase; AKe, AKst, and AKy, *Escherichia coli, Bacillus stearothermophilus*, and yeast adenylate kinase; AK1, muscle cytosolic adenylate kinase; AK2 and AK3, mitochondrial adenylate kinases; Ap_5A, P^1,P^5-di(adenosine-5′) pentaphosphate; blue-Sepharose, Cibacron blue 3G-A Sepharose CL-6B; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PMPS, *p*-(hydroxymercuri)benzenesulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone.

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The vectors used to clone and sequence the *B. stearothermophilus adk* gene and to overproduce its protein product were either pUC18 or pUC19 (Yanish-Perron et al., 1985). DNA sequencing was performed by the dideoxynucleotide sequencing method (Sanger et al., 1977) on double-strand DNA (Zhang et al., 1988).

Purification of AK and Activity Assays. The wild-type enzyme from the AKe-overproducing strain of *E. coli* was purified as described previously (Bârzu & Michelson, 1983). The AKst overproduced in *E. coli* was purified by essentially the same procedure, which involves chromatography on blue-Sepharose and Ultrogel AcA54. Prior to chromatography, the bacterial extract was heated at 65 °C for 20 min. AK activity was determined in both directions with the coupled spectrophotometric assay (Saint Girons et al., 1987). At temperatures higher than 45 °C, an end-point procedure was used. The reaction mixture (0.1 mL), which contained 50 mM Tris-HCl (pH 7.4), 4 mM $MgCl_2$, 2 mM ATP, 2 mM AMP, and 0.25 M KCl, was incubated with AKst (between 15 and 60 ng) for 2–10 min. Subsequently, 0.05 mL of this mixture was added to 0.45 mL of medium containing 50 mM Tris-HCl (pH 7.4), 6 mM $MgCl_2$, 30 mM KCl, 0.5 mM phosphoenolpyruvate, 0.25 mM NADH, 0.1 mM Ap_5A, and 2 units of lactate dehydrogenase. After the absorbance was measured at 334 nm, 2 units of pyruvate kinase was added to this mixture, and the absorbance (stabilized in less than 1 min) was measured again. This assay, which did not require the use of coupling enzymes at temperatures above 45 °C, was simple and reproducible (±5% error). One unit of AK corresponds to 1 μmol of product formed per minute at a given temperature.

Spectroscopic Methods. Circular dichroism spectra were recorded on a Jasco-600 spectropolarimeter using quartz cylindrical cells of 0.2-mm pathlength and a protein concentration of approximately 0.4 mg/mL. The equilibrium unfolding induced by guanidine hydrochloride was monitored at 20 °C by following the ellipticity at 222 nm as described previously (Rose et al., 1991b). ^1H NMR experiments were performed on a Varian Unity 500-MHz spectrometer equipped with a SUN SPARC+ computer. The spectra were recorded at several temperatures with a 2-s relaxation delay, during which the residual water signal was suppressed with a shaped pulse.

Differential Scanning Calorimetry. The thermal stability of AKe and AKst was studied by DSC using an ultrasensitive Microcal MC-2D instrument at a scanning rate of approximately 50 °C/h. Measurements were performed at a protein concentration of approximately 2 mg/mL in 50 mM HEPES buffer (pH 7.2). Calorimetric data were analyzed with the software provided by Microcal, Northampton, MA.

Other Analytical Procedures. Protein concentration was determined according to Bradford (1976) using a Bio-Rad kit. SDS–PAGE was performed as described by Laemmli (1970). Trypsin digestion, peptide separation by HPLC, and amino acid analysis were performed as described previously (Saint Girons et al., 1987). Elemental analysis was performed using a multielement scan inductively coupled plasma mass spectrometer. Zn^{2+} was subsequently quantitated by graphite furnace atomic absorption. Parallel experiments were performed with AKe as a reference. Protein samples were dissolved in a minimum volume of ultrapure water containing 2% high-purity HNO_3.

Results

Molecular Cloning, DNA Sequencing, and Overexpression of the B. stearothermophilus adk Gene. B. stearothermophilus chromosomal DNA was partially cleaved by the restriction enzyme *Sau*3A and ligated to pUC19 DNA linearized by *Bam*HI. *E. coli adk* thermosensitive strain CR341 T28 was transformed with the ligation mix and plated on LB plates containing ampicillin (100 mg/L) and thymine (0.01%) at 42 °C. One clone growing at this temperature was further analyzed. Plasmid DNA from this strain (pDIA5308) was purified. A restriction map of the plasmid pDIA5308 is presented in Figure 1. The *adk* gene on the 5.5-kb fragment was localized, utilizing the previously established sequence of the *B. subtilis* L30 operon (Nakamura et al., 1990), which contains the *adk* gene, and assuming that the organization of the operon would be conserved between the two bacilli. The chromosomal insert was first sequenced from both ends using the universal primer and the reverse primer. Then, the three *Hin*cII fragments and the *Eco*RI fragment were subcloned in pUC18, and the sequence from both extremities of each fragment was determined. The sequences obtained were compared to the sequence of the *B. subtilis* L30 operon. This enabled us to precisely localize the *adk* gene (see Figure 1). Furthermore, we also identified the *sec*Y gene upstream of the *adk* gene and the 5′-terminal region from the *map* gene downstream. The complete nucleotide sequence of the *adk* gene was determined after subcloning in the plasmid pUC19 and use of synthetic oligonucleotides (Figure 2). The predicted sequence of the AKst, the C-terminal portion of the *sec*Y gene product, and the N-terminal part of the methylaminopeptidase are also shown. The methionine initiation codon was proposed after comparison with other AKs. The organization of the gene is highly conserved between *B. subtilis* and *B. stearothermophilus*.

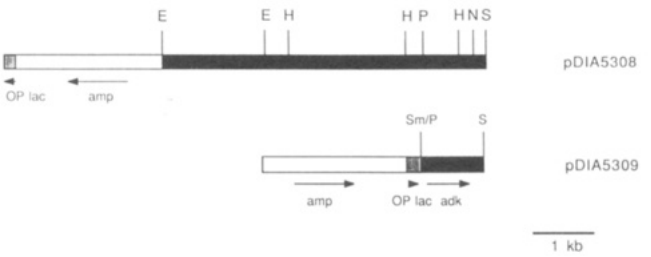

FIGURE 1: Restriction maps of plasmids pDIA5308 and pDIA5309. The inserted *B. stearothermophilus* DNA is black. Restriction sites are represented as follows: E, *Eco*RI; H, *Hin*dII; N, *Nco*I; P, *Pst*I; S, *Sal*I; Sm, *Sma*I.

The expression of the *adk* gene in plasmid pDIA5308 is relatively low (approximately 1% of total *E. coli* proteins). This expression probably derived from the activity of the native promoter of the S30 operon. To overproduce AKst, the *Pvu*II–*Sal*I restriction fragment encompassing the *adk* gene was cloned into the *Sma*I–*Sal*I restriction sites of the vector pUC18 (Figure 1). This construct, named pDIA5309, allowed the overexpression of the *adk* gene under the control of the *lac* promoter.

Molecular and Kinetic Properties of AKst. Purified AKst (M_r = 24135) showed an apparent molecular mass of 25 kDa on SDS–PAGE. The amino acid composition of the enzyme and of the tryptic peptides resolved by reverse-phase HPLC was found to be in full agreement with that predicted from the nucleotide sequence of the *adk* gene.

In searching for optimal assay conditions, we found that AKst requires a higher ionic strength (250 mM KCl) than AKe (50 mM). Table I shows the kinetic parameters of AKst determined at 37 °C, compared to those of AKe determined at 30 °C. The K_m for nucleotide substrates was similar for AKst and AKe, except for a 10-fold lower K_i of AKst for Ap_5A. Excess AMP (above 0.14 mM) inhibited the activity

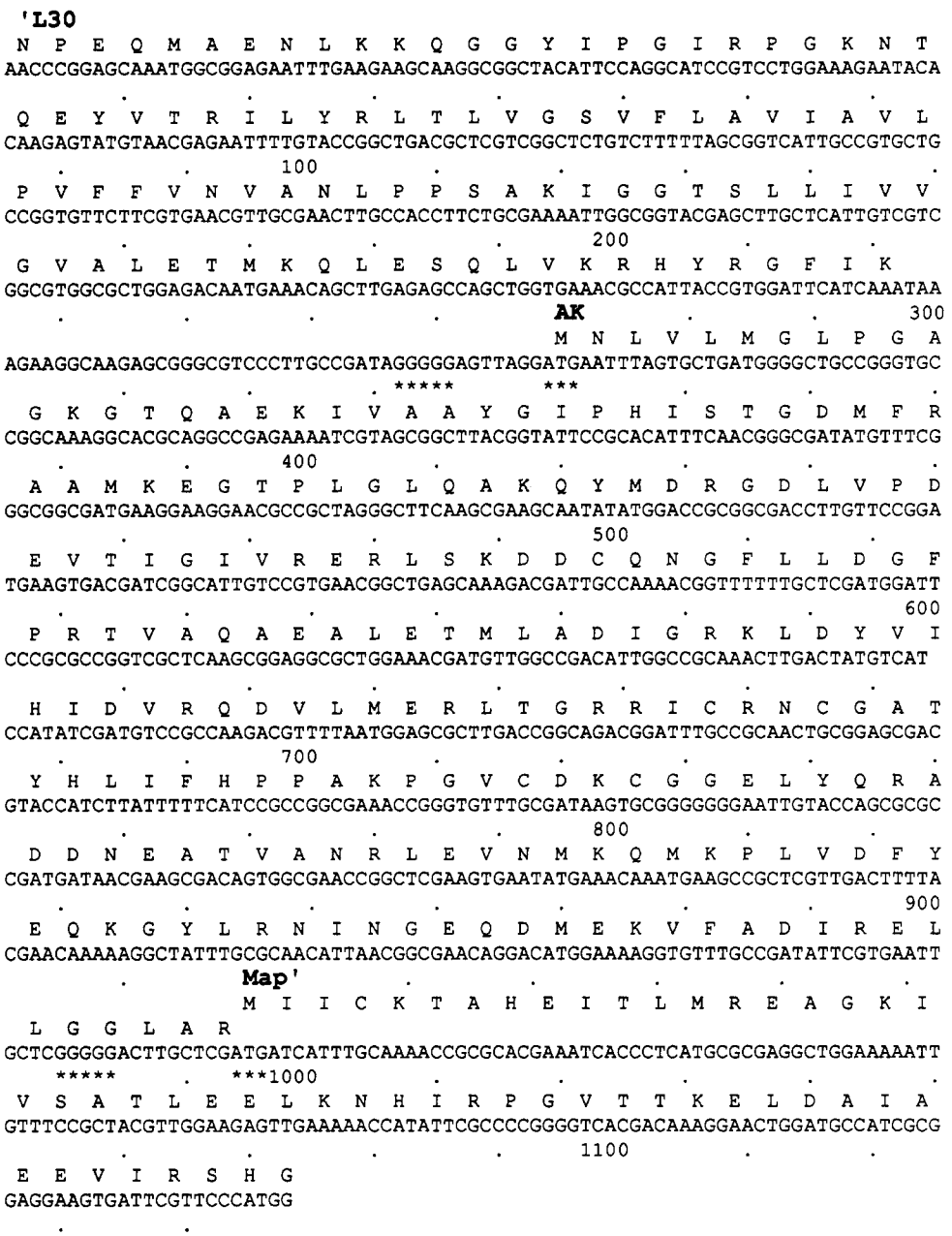

FIGURE 2: Nucleotide and deduced amino acid sequences of the 1.1-kb *Hin*dII *Nco*I fragment. Nucleotides are numbered from the 5′ end. The ribosome putative binding sites and the initiation codons are labeled with asterisks.

Table I: Kinetic Parameters of AKe (at 30 °C) and of AKst (at 37 °C)[a]

adenylate kinase	K_m(ADP) (μM)	K_i(Ap_5A) (μM)	V_{max}(ADP) [μmol/(min·mg of protein)]	K_m(ATP) (μM)	K_m(AMP) (μM)	V_{max}(ATP,AMP) [μmol/(min·mg of protein)]
E. coli	92	0.6	605	51	38	1020
B. stearothermophilus	37	0.06	225	36	76	288

[a] The reaction medium (0.5-mL final volume) contained either 50 mM Tris-HCl, pH 7.4, 50 mM (AKe) or 250 mM (AKst) KCl, 1 mM glucose, 0.4 mM $NADP^+$, 2 mM $MgCl_2$, different concentrations of ADP (in the absence or presence of Ap_5A), and 2 units each of hexokinase and glucose-6-phosphate dehydrogenase or 50 mM Tris-HCl, pH 7.4, 0.2 mM NADH, 0.5 mM phosphoenolpyruvate, 50 mM (AKe) or 250 mM (AKst) KCl, 3 mM $MgCl_2$, and 2 units each of lactate dehydrogenase and pyruvate kinase. The reaction was started with enzymes diluted at 2–30 μg/mL in 50 mM Tris-HCl, pH 7.4. K_m(ADP), K_i(Ap_5A), and V_{max}(ADP) were determined from plots of $1/v$ versus $1/ADP^2$, which assumes that the two molecules of ADP bind to the enzyme with the same affinity. The apparent K_m for ATP and for AMP was determined at a single fixed concentration of cosubstrates (1 mM ATP and 0.14 mM AMP, respectively). The V_{max}(ATP,AMP) was obtained by extrapolating the reaction rates for infinite concentrations of ATP and AMP and assuming that the concentration of one nucleotide substrate does not affect the apparent K_m for the second nucleotide substrate.

of AKst as well as that of AKe (Saint Girons et al., 1987; Liang et al., 1991). AK activity determined at various temperatures (between 20 and 45 °C for the *E. coli* enzyme and between 20 and 65 °C for the *B. stearothermophilus* enzyme) showed linear Arrhenius plots. The calculated ΔH^* values were of 11 kcal/mol for AKe and 17.4 kcal/mol for AKst. At optimal temperatures (45 °C for AKe and 65 °C for AKst) the specific activities of two isoforms are very similar (2400 units/mg for AKe and 3000 units/mg for AKst).

Identification of Tightly Bound Zn^{2+} as a Structural

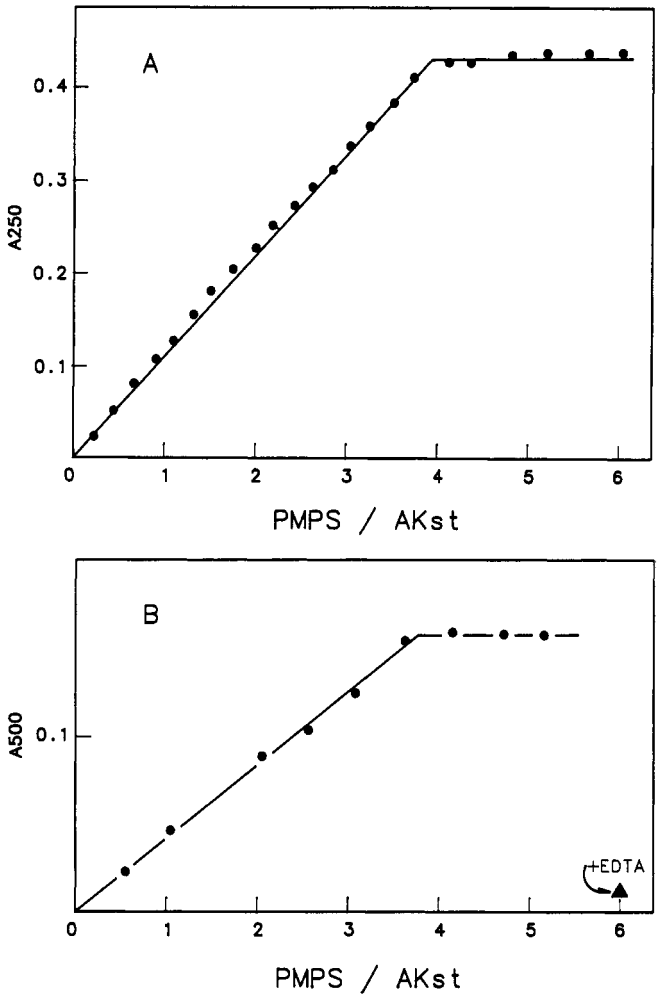

FIGURE 3: Titration of native AKst with PMPS. (A) AKst (29 μM in 0.6 mL of 10 mM Tris-HCl, pH 8) was treated with PMPS (1 mM solution in the same buffer) to give the indicated molar ratios of PMPS to enzyme. The absorption at 250 nm, indicative of the formation of the S^- Hg^{2+} charge-transfer complex, was measured relative to the control value at the beginning of the titration. (B) A titration was also performed in the presence of the zinc-binding dye 4-(2-pyridylazo)resorcinol (0.1 mM). The absorption developed at 500 nm reflects formation of a zinc–dye complex. The value at the saturation level corresponds to 0.83 mol of Zn^{2+} released/mol of AKst. (▲) indicates absorbance after addition of 2 mM EDTA.

Component of AKst. Out of five cysteines in AKst, only one, presumably Cys77, reacted with DTNB. Furthermore, this thiol group was accessible to DTNB only under denaturing conditions, resembling closely the behavior of a single Cys in AKe (Saint Girons et al., 1987). The spacing of the remaining four Cys residues in AKst, Cys130, Cys133, Cys150, and Cys153 (Figure 2), shows striking similarity to that found in a number of nucleic acid binding proteins which are able to coordinate a structural zinc atom in a zinc finger-like fashion (Evans & Hollenberg, 1988; Vallee & Auld, 1990). Indeed, analysis of AKst for metal ions by atomic absorption spectrophotometry revealed the presence of approximately 0.8 mol of zinc/mol of protein. No significant amount of zinc was found in AKe. The involvement of four cysteines in coordination of zinc was confirmed by titration of AKst with PMPS, a strongly dissociating sulfhydryl reagent (Hunt et al., 1984; Giedroc et al., 1986). The titration, in which formation of the PMPS-sulfhydryl chromophore was followed at 250 nm, indicated linear incorporation of PMPS up to approximately 3.9 mol of reagent/mol of AKst (Figure 3A). Binding of PMPS was accompanied by the release of Zn^{2+}. This is demonstrated by the titration with 4-(2-pyridylazo)resorcinol,

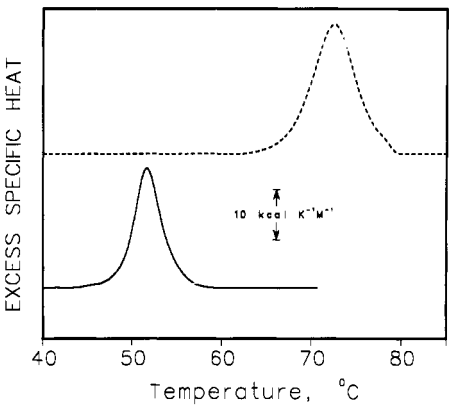

FIGURE 4: Excess heat capacity curves for AKst (upper trace) and AKe (lower trace). The curves were obtained by smoothing raw differential scanning calorimetric traces and subtraction of the corresponding lines using a cubic spline interpolation procedure.

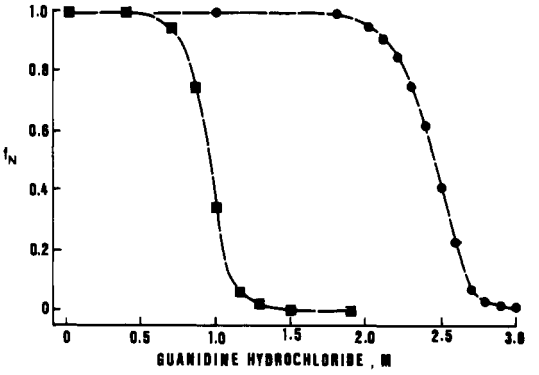

FIGURE 5: Dependence of the apparent fraction of folded protein, f_N, on the concentration of guanidine hydrochloride: (●) AKst; (■) AKe.

a zinc-binding dye which upon metal coordination develops absorption at 500 nm (Hunt et al., 1984). A linear release of Zn^{2+} up to approximately 4 equiv of PMPS added to the AKst solution was observed (Figure 3B). The color development corresponded to 0.83 mol of zinc released/mol of enzyme. These data provide strong indication that the structural zinc atom in AKst is bound to four sulfur atoms, most likely of Cys130, Cys133, Cys150, and Cys153.

Thermodynamic Stability of AKst. The excess heat capacity curve for AKst is shown in Figure 4 (upper trace), together with a previously published curve (Rose et al., 1991b) for AKe (lower trace). These curves were obtained by subtracting the base lines from raw DSC data using a cubic spline interpolation procedure. The midpoint denaturation temperature (T_m) for AKst is 74.5 °C, and the calorimetric enthalpy of denaturation (ΔH_{cal}) amounts to 145 kcal/mol. This may be compared with a T_m of 51.8 °C and a ΔH_{cal} of 95 kcal/mol for AKe. The increased thermodynamic stability of AKst is also indicated by the results of equilibrium unfolding experiments in guanidine hydrochloride (Figure 5). For AKst the midpoint transition concentration of guanidine hydrochloride is 2.45 M, while the respective number for AKe is 0.93 M. In parallel with circular dichroism and differential scanning calorimetry measurements, the low-field region of the 1D NMR spectrum of AKst was recorded at various temperatures (not shown). Several signals from slowly exchanging amide protons and His aromatic C_2H protons appeared in the spectrum at 25 °C. The chemical shift dispersion observed in this region of the spectrum is characteristic of folded proteins. When the temperature was increased to 70 °C, changes in the intensity and chemical shift of some peaks were observed; however, a high proportion of the native-like

A

Source	Homologous Sequence	Reference
B. stearotherm.	^{127}RRI**C**RN**C**GATYHLIFHPPAKPGV**C**DK**C**GG-ELYQRADDN^{164}	this work
L. lactis	RYI**C**RN**C**GATYHKIFNPTKVEGT**C**DV**C**GSHDLYQRADDV	a
B. subtilis	RRI**C**SV**C**GTTYHLVFNPPKTPGI**C**DK**D**GG-ELYQRADDN	b
M. capricolum	RLV**C**PL**C**KASFNLETRKPKQEGL**C**DF**D**NT-KLVKRSDDS	c
P. denitrif.	RFT**H**GN**C**GEVYGDVTKPTKEPGK**C**DV**C**GSTDLRRRADDN	d
E. coli	RRV**H**APSGRVYHVKFNPPKVEGK**D**DVTGE-ELTTRKDDQ	e
H. influenza	RRV**H**QASERSYHIVYNPPKVEGK**D**DVTGE-DLIIRADDK	f
Yeast	RLI**H**PASGRSYHKEFNPPKEDMK**D**DVTGE-ALVQRSDDN	g
AK2	RLI**H**PQSGRSYHEEFNPPKEDMK**D**DETGE-PLIRRSDDN	h
AK3	RWI**H**PGSGRVYNIEFNPPKTMGI**D**DLTGE-PLVQREDDR	i
AK1	^{132}R--GETSGRV----------DDN^{142}	j

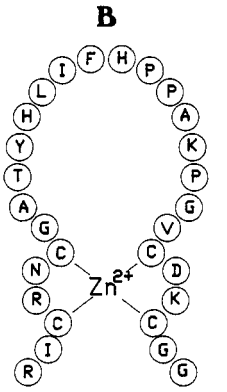

FIGURE 6: Alignment of partial amino acid sequences in 11 isoforms of adenylate kinase (A) and schematic representation of the zinc finger-like structure (B) in AKst (residues 128–155). The conserved Cys residues (or equivalent His/Asp residues) are shown in boldface letters. References: (a) Koivula & Hemilä, 1991; (b) Nakamura et al., 1990; (c) Ohkubo et al., 1987; (d) Spürgin et al., 1989; (e) Brune et al., 1985; (f) Maskell et al., 1991; (g) Tomasselli et al., 1986a; (h) Frank et al., 1986; (i) Tomasselli et al., 1986b; (j) Heil et al., 1974.

spectrum was still seen at this temperature. Consistent with its higher thermodynamic stability, AKst was found considerably more resistant to trypsin inactivation than AKe. At a trypsin/AK ratio of 1/50 and 37 °C, the first-order rate constant of inactivation (k_1) for AKe was 7.4×10^{-3} s^{-1}, compared with a k_1 value of 0.9×10^{-3} s^{-1} for AKst.

Discussion

A key finding of this study is the observation that adenylate kinase from *B. stearothermophilus* contains a tightly bound zinc atom. Although the crystal structure of the protein is yet unknown, all experimental evidence points to the coordination of this zinc atom by four cysteines in a manner common to a whole class of "zinc finger" proteins (Klug & Rhodes, 1987; Vallee & Auld, 1990). In analogy with the crystal structure of AK1 (Dreusicke et al., 1988), two of these cysteines (Cys130 and Cys133) are likely to be involved in reverse turn. The remaining two Cys residues (Cys150 and Cys153) belong to a 25 amino acid long segment that is missing in the structurally characterized AK1 but is present in all known bacterial AKs as well as in the mammalian mitochondrial isoforms AK2 and AK3 (Schultz et al., 1986).

No zinc-binding properties have been reported hitherto for other adenylate kinases. However, primary structures within the adenylate kinase family show a high degree of similarity. Alignment of the sequences from ten "long" isoforms of AK reveals intriguing homologies in the region comprising four cysteines in AKst (Figure 6A). Thus, Cys130 is conserved in three other bacterial enzymes and is substituted with His in all remaining long variants. Cys133, common to five bacterial AKs, is substituted with Ser in the remaining isoforms. Cys150 is also common to five bacterial AKs, while it is replaced by Asp in AKe, AK of *Hemophilus influenza*, AKy, AK2, and AK3. Finally, Cys153 is only conserved in AKst and the AKs from *Paracoccus denitrificans* and *Lactococcus lactis*. It is substituted with Asp in the enzymes from *B. subtilis* and *Mycoplasma capricolum* and with Thr in the remaining isoforms. It is well documented that the affinity of zinc for His and Glu/Asp is nearly equal to that for Cys (Vallee & Auld, 1990). It seems thus legitimate to predict that each of the first five AKs depicted in Figure 6A will be able to coordinate zinc and to form structures resembling those known as zinc fingers (Figure 6B). The experimental observation that AKe does not contain Zn^{2+} is not surprising since in this enzyme the tetrahedral coordinating ability of zinc is lost. Similarly, no zinc-binding ability is predicted for AKy, AK2, AK3, AK1, and AK from *H. influenza*.

Complexation of zinc is likely to rigidify certain regions of the protein and thus may contribute to the high thermodynamic stability of AKst. Indeed, removal of the bound zinc by treatment of AKst with PMPS, EDTA, and dithiothreitol decreased the denaturation temperature of the enzyme from 74.5 °C to approximately 67 °C (data not shown). However, the latter temperature is still much higher than the unfolding temperature of AKe (51.8 °C). It thus appears that, in addition to metal binding, other factors such as an unusually high ratio of Arg/(Arg + Lys) residues (Argos, 1989) also contribute to the enhanced stability of AKst.

A question arises whether the presence of a zinc finger-like motif in AKst (and likely in some other isoforms of adenylate kinase) has functional consequences unrelated to the known catalytic activity of the enzyme. In a number of other proteins, zinc fingers have been implicated in the recognition and binding of DNA (Vallee & Auld, 1990). In this context, we recall experimental observations which suggest a more complex biological role of adenylate kinase than is generally recognized. The functional role attributed to AK includes the modulation of phospholipid (Goelz & Cronan, 1982) or macromolecular (Huss & Glaser, 1983) biosynthesis and thus involvement in the control of cell growth. The relation of the zinc finger-like motif in some AK isoforms to these putative functions remains to be explored.

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