Zinc Chelation and Structural Stability of Adenylate Kinase from *Bacillus subtilis*†

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ABSTRACT: Adenylate kinase from *Bacillus subtilis*, like the enzyme from *Bacillus stearothermophilus*, contains a structural zinc atom. Cys153 in the enzyme from *B. stearothermophilus*, which is involved in the zinc coordination, is replaced in the adenylate kinase from *B. subtilis* by an aspartic acid residue. Therefore, we were interested in establishing whether this difference has an impact on the structure, the metal chelation, and the overall stability of these proteins. We also were interested in determining whether His138, which is conserved in many adenylate kinases, can act as a fourth partner in the metal chelation and, in general, whether His can successfully replace Cys or Asp in coordinating zinc in the adenylate kinase from *B. subtilis*. The *adk* gene from *B. subtilis* was cloned by polymerase chain reaction. The wild-type protein, together with several variants obtained by site-directed mutagenesis, were expressed in *Escherichia coli* and analyzed by biochemical and physicochemical methods. The H138N and D153C mutants of adenylate kinase from *B. subtilis* exhibited properties similar to those of the wild-type protein, indicating that His138 is not involved in metal coordination and that Asp153, just like Cys in the analogous position in the enzyme from *B. stearothermophilus*, can participate in zinc chelation. This is the first experimental evidence indicating that aspartic acid can be involved in the coordination of a structural zinc atom. On the other hand, the D153H and D153T variants showed significant changes in their zinc-binding properties. Dialysis of the latter proteins against buffer (in both the presence and the absence of 2 mM EDTA) resulted in removal of the metal ion and loss of enzymatic activity. These mutants reacted readily with 5,5′-dithiobis(2-nitrobenzoic acid) under "native" conditions, unlike the wild-type protein and the H138N and D153C variants of the enzyme. A fifth modified form of adenylate kinase from *B. subtilis* probed in this study, C130H, showed characteristics similar to those of the D153T mutant with respect to both metal chelation and reactivity toward 5,5′-dithiobis(2-nitrobenzoic acid). Differences between the circular dichroism spectra of wild-type enzyme and those of the C130H mutant suggest a less compact structure of the mutant, which also explains its decreased stability against denaturation by temperature or guanidinium hydrochloride or against inactivation by trypsin. In conclusion, the zinc-chelating property of adenylate kinase from *B. subtilis*, and in general from the Gram-positive bacteria, is compatible only with the presence of three or four Cys residues in the following sequence: Cys-X_2-Cys-X_{16}-Cys-X_2-Asp/Cys.

Adenylate kinase (AK,[1] ATP:AMP phosphotransferase, EC 2.7.4.3) is a key enzyme involved in the energy metabolism of prokaryotic and eukaryotic cells (Noda, 1973). The protein has been extensively used as a model for studying structure–function relationships in the whole class of kinases (Tsai & Yan, 1991). The small size and the availability of a large number of variants render adenylate kinase particularly well suited for identification of specific residues involved in nucleotide binding and catalysis, as well as those critical for maintaining structural stability of the protein.

Bacterial adenylate kinases as well as the enzymes from yeast (AK_y) or mammalian mitochondria (AK2 and AK3) belong to the class of "long" forms. They differ from the short variant (AK1) by a 25-residue-long insertion which remains relatively well conserved among various species (Schulz, 1987). Structural studies with AK from *Escherichia coli* (AK_e) indicate that a large portion of this extra sequence is involved in β-strands (Müller & Schulz, 1992). Recently, we have shown that AK from *Bacillus stearothermophilus* (AK_{st}) contains as an essential structural element a zinc atom

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[1] Abbreviations: AK, adenylate kinase; AK1, muscle cytosolic adenylate kinase; AK2 and AK3, mitochondrial adenylate kinases; AK_y, AK_{st}, AK_{sub}, and AK_e, yeast, *B. stearothermophilus*, *B. subtilis*, and *E. coli* adenylate kinase; Blue-Sepharose, Cibacron Blue 3G-A Sepharose CL-6B; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PAR, 4-(2-pyridylazo)resorcinol; PMPS, *p*-(hydroxymercuri)benzenesulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone; lb, line broadening; gb, Gaussian broadening.

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coordinated by four cysteine residues (Glaser *et al.*, 1992). Screening 25 other bacteria which differ in their optimum growth conditions, we found that AKs from Gram-positive bacteria contain Zn^{2+}. In contrast, enzymes from Gram-negative species are all devoid of the metal atom (Gilles *et al.*, 1994). Comparison of the primary structure of AK_{st} with that deduced from the nucleotide sequence of the *adk* gene from *Bacillus subtilis*, a Gram-positive mesophile, revealed 76% identity. However, one of the cysteines essential for zinc chelation in AK_{st} (Cys153) is replaced in AK_{sub} by Asp. This is somewhat unexpected since carboxylate oxygens are believed to be involved in coordination of catalytic but not structural zinc atoms (Vallee & Auld, 1990, 1993). The above observation prompted us to hypothesize that one of the two His residues in AK_{sub}, and particularly His138, may act as the fourth partner in the metal chelation. In order to test this hypothesis and to probe the role of various residues in zinc chelation and maintenance of the structural integrity of the protein, we have cloned the *adk* gene from *B. subtilis*, the nucleotide sequence of which had been determined previously (Nakamura *et al.*, 1990). The protein and a number of variants obtained by site-directed mutagenesis were expressed in *E. coli* and analyzed by biochemical and physicochemical methods. Experimental data argue against the involvement of His138 in zinc chelation and show that neither His138 → Asn or Asp153 → Cys substitution has any effect on the metal-binding properties of AK_{sub} or on the structural properties and thermodynamic stability of the enzyme. This may be contrasted with the substitution of Cys130 with His, which impairs the zinc-binding function of the enzyme, perturbs its secondary structure, and lowers the thermodynamic stability of the protein and its resistance to proteolytic digestion. An impaired zinc-binding function and an increased susceptibility to proteolytic inactivation were also observed for the Asp153Thr mutant.

MATERIALS AND METHODS

Chemicals. Adenine nucleotides, restriction enzymes, T4 DNA ligase, T4 DNA polymerase, and coupling enzymes were from Boehringer Mannheim. Taq DNA polymerase was from Cetus Corporation. Blue-Sepharose was from Pharmacia LKB Biotechnologies Inc. Oligonucleotides were synthesized according to the phosphoramidate method using a commercial DNA synthesizer (Cyclone TM Biosearch). Deuterated water (99.8%) was from Eurisotop (Gif-sur-Yvette), and deuterated phosphate was from Merck (Darmstadt).

Bacterial Strains, Plasmids, Growth Conditions, and DNA Manipulations. *B. subtilis* strain 168 was used to clone the *adk* gene. The bacterial chromosomal DNA was prepared as previously described (Saunders *et al.*, 1984). The XL1 Blue *E. coli* strain (Bullock *et al.*, 1987) was used for DNA sequencing. Uracil-containing single-stranded DNA was produced by the ung dut *E. coli* strain RZ1032 (Kunkel *et al.*, 1985). *B. subtilis adk* gene carried by the plasmid pDIA5316 was overexpressed in the FB8 *E. coli* strain (F. Blasi) under the control of the *lac* promoter. For overexpression of the *B. subtilis adk* gene, *E. coli* strains harbor a second plasmid, pDIA17, which encodes the *lacI* gene (Munier *et al.*, 1991). *E. coli* strains were grown in LB medium (Miller, 1972) supplemented with 100 mg/L ampicillin, 70 mg/L kanamycin, and 20 mg/L chloramphenicol when required. The *lac* promoter was induced with 1 mM β-D-thiogalactoside when the culture reached an absorbance of 0.5 at 600 nm. Bacteria were harvested by centrifugation 4 h after induction.

Polymerase Chain Reaction (PCR), Site-Directed Mutagenesis, and Sequence Analysis. The *B. subtilis adk* gene is part of the L30 operon (Nakamura *et al.*, 1990). In order to overexpress it, the complete *adk* gene was amplified by PCR (Sambrook *et al.*, 1989) and cloned in the expression vector pTZ19 (Pharmacia). The two synthetic oligonucleotides used for amplification were 5′-A C **G G A T C C** T A T G A A A A A C T A G A G G-3′ and 5′-A C **G A A T T** C A T T T T T T T A A T C C T C C-3′. During the amplification an *Eco*RI and a *Bam*HI restriction site (in bold in the oligonucleotide sequences) were created at both ends of the amplified fragment. After digestion by these two enzymes, the amplified *adk* gene was inserted into the pTZ19 plasmid digested by the same enzymes. Six clones containing the *B. subtilis adk* gene and overexpressing the protein were characterized, and one of them (pDIA5316) was kept for further studies. Site-directed mutagenesis was performed on the single-stranded form of plasmid pDIA5316 grown in strain RZ1032 in the presence of plasmid pDIA17 and the helper phage M13 K07 (Dotto & Zinder, 1984). The Cys (TGC) codon at position 130 was modified to a His (CAC) codon using the oligonucleotide 5′-CGCAGAATTCACAGTG-TTTGCGGC-3′. The His (CAT) codon at position 138 was modified to a Asn (AAT) codon using the oligonucleotide 5′-ACAACCTATAATTTAGTCT-3′. The Asp (GAC) codon at position 153 was modified to a Cys (TGT), Thr (ACC), or His (CAC) codon using the oligonucleotides 5′-TTGT-GATAAATGTGGAGGCGAAC-3′, 5′-TTGTGATAAAAC-CGGAGGCGAAC-3′, and 5′-TTGTGATAAACACGGAG-GCGAAC-3. The absence of any mutational events in the course of the amplification or of a second mutation during the site-directed mutagenesis was verified by the dideoxynucleotide sequencing method (Sanger *et al.*, 1977).

Purification of AK and Activity Assay. The adenylate kinases overexpressed in *E. coli* were purified as described previously (Bârzu & Michelson, 1983) by a two-step procedure involving chromatography on Blue-Sepharose and Ultrogel AcA54. Adenylate kinase activity was determined at 30 °C using the coupled spectrophotometric assay at 334 nm (0.5-mL final volume) on an Eppendorf PCP6121 photometer (Saint Girons *et al.*, 1987). One unit of enzyme activity corresponds to 1 μmol of the product formed in 1 min at 30 °C and pH 7.4. Apo-AK_{sub} (wild-type and mutant forms) was prepared by addition of a slight excess of *p*-(hydroxymercuri)phenylsulfonate (PMPS) over the protein. One millimolar EDTA was then added, and the formation of PMPS–AK complex was reversed by addition of 2 mM dithiothreitol. The protein solution was immediately desalted onto a Sephadex G-25 column equilibrated in 50 mM Tris-HCl, pH 7.4. The resultant proteins prepared in similar fashion were found to contain less than 0.05 mol of Zn^{2+} per mole of protein.

Differential Scanning Calorimetry. The thermal stability of the wild-type AK_{sub} and the mutant proteins was studied by differential scanning calorimetry using an ultrasensitive Microcal MC-2D instrument at a scanning rate of approximately 50 °C/h. Protein concentrations were in the range of 1–1.5 mg/mL in 50 mM Tris-HCl buffer, pH 7.2. Data from DSC experiments were analyzed by employing the software provided by Microcal Inc., Northampton, MA.

NMR Measurements. NMR samples were obtained by dissolving the lyophilized protein in phosphate buffer (100 mM, pH 6.55) in ^2H_2O at an approximate concentration of 0.2 mM. One half of the solution was used for titration at basic pH (using small aliquots of diluted NaOD), and the other half was used for measurements at lower pH values.

The NMR spectra were recorded at 310 K on a Varian UNITY500 NMR spectrometer equipped with an ultrashim device. Residual water signal was eliminated by irradiation during the relaxation delay (1.2 s). Usually, 512 or 1024 scans were necessary to obtain a good signal-to-noise ratio. Processing of time-dependent signals was done with FELIX software (Denis Hare Inc.). A Gaussian multiplication (lb = –2 Hz; gb = 0.15) prior to Fourier transformation was found to be a good compromise between resolution and sensitivity. Proton chemical shifts are expressed in parts per million (ppm) from a residual HDO signal, which is 4.74 ppm downfield from 2,2-dimethyl-5-silapentane-5-sulfonate.

Infrared Spectroscopy. Infrared absorbance spectra were recorded on a Digilab FTS-40A FT-IR spectrometer with a liquid nitrogen cooled MCT detector. Protein solutions in D_2O buffer were placed between a pair of CaF_2 windows separated by a path length of 45 μm. For each sample, 512 interferograms were co-added and Fourier-transformed to generate spectra with a nominal resolution of 2 cm^{-1}. Infrared difference spectra were obtained by using software developed in-house.

Circular Dichroism. CD spectra were recorded on a Jasco-600 polarimeter using quartz cylindrical cells of 0.2 mm and a protein concentration of about 15 μM. The equilibrium unfolding as a function of guanidine-HCl was monitored at 20 °C by measuring ellipticity at 222 nm. Before measurements, samples in the presence of guanidine-HCl were equilibrated for approximately 12 h at room temperature. The fraction of folded protein, f_N, was calculated as $f_N = (\theta - \theta_u)/(\theta_N - \theta_u)$, where θ is the observed ellipticity and θ_N and θ_u are the values of ellipticity for the native and totally unfolded forms, respectively. Values for θ_N and θ_u in the transition zone were determined by linear extrapolation.

Other Analytical Procedures. Protein concentration was measured according to Bradford (1976), using pure AK as a calibration standard, or by amino acid analysis (for quantitative CD spectroscopy and DSC experiments). Amino acid analysis was performed on a Beckman System 6300 high-performance analyzer after 6 N HCl hydrolysis for 22 h at 110 °C. SDS–polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). Zinc content of AK_{sub} was determined by atomic absorption spectrophotometry (Glaser *et al.*, 1992).

RESULTS

Overexpression, Purification, and Zinc Content of AK_{sub}. Plasmid pDIA5316 and derivatives resulting from site-directed mutagenesis overproduced AK_{sub} under the control of the *lac* promoter. The protein represents 4–5% of the total *E. coli* protein. Chromatography on Blue-Sepharose and elution with 1 mM ATP + 1 mM AMP yielded >80% pure protein. Subsequent chromatography on Ultrogel AcA54 yielded a homogenous preparation of AK_{sub} (Figure 1). AK_{sub} (calculated molecular mass, 24.1 kDa), like AK_e, exhibits an anomalous migration on SDS–PAGE (apparent molecular mass, 27.5 kDa). The amino acid composition of AK_{sub} was found to be in good agreement with that expected from the nucleotide sequence of the corresponding gene (data not shown). The specific activity of wild-type AK_{sub} and those of its mutants varied between 350 and 400 units/min/(mg of protein) at 30 °C and pH 7.4 (in the direction of ATP formation). The activities of the wild-type protein and the H138N and D153C mutants remained stable for weeks when the enzymes were kept as frozen solutions or lyophilized powders. In contrast, the C130H, D153H, and D153T mutants lost over 80% of their activity after 24 h of dialysis against 50 mM ammonium bicarbonate, pH 7.8. For this reason the last three modified forms of AK_{sub} were analyzed for their properties shortly after chromatography on Ultrogel AcA54.

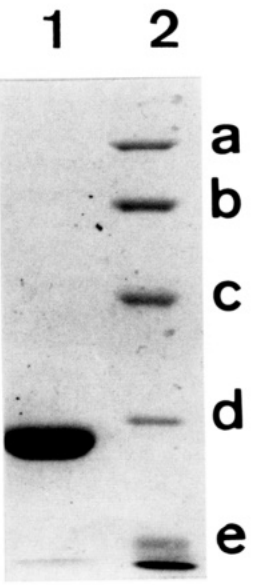


FIGURE 1: SDS–PAGE (10%) of AK_{sub}. Lane 1, pure enzyme after Ultrogel AcA54 chromatography (10 μg of protein). Lane 2, standard proteins, from top to bottom: a, phosphorylase a (94 000); b, bovine serum albumin (67 000); c, ovalbumin (43 000); d, carbonic anhydrase (30 000); e, soybean trypsin inhibitor (20 100).

Analysis of AK_{sub} for metal ions by atomic absorption spectrophotometry revealed the presence of 0.8–1 mol of zinc per mole of protein (wild type and H138N, D153C, and D153H mutants). This number was significantly lower in the case of C130H (0.34 mol of zinc per mole of protein) and D153T (0.60 mol of zinc per mole of protein) mutants. Moreover, after 24 h of dialysis against 50 mM Tris-HCl (pH 7.4) buffer, the zinc:protein molar ratio was reduced to 0.07 in the case of the C130H mutant and to 0.38 in the case of the D153T mutant, whereas in the case of the wild-type enzyme and the H138N and D153C mutants it remains unchanged.

Reaction of AK_{sub} with DTNB and PMPS. The four Cys residues in the wild-type AK_{sub} are situated at positions 77, 130, 133, and 150. The first of these cysteines is located in the same position as the single Cys residue in AK_e. This residue, buried in AK_e, reacted with DTNB only in the presence of urea at concentrations higher than 2 M (Saint Girons *et al.*, 1987). Under native conditions both the wild-type AK_{sub} and the D153C and H138N mutants reacted very slowly with DTNB (<0.1 SH groups/mole of enzyme during a 10-min period at room temperature and in the presence of a 5-fold excess of DTNB over the protein), indicating that none of the four (wild type and H138N) or five (D153C) Cys groups are readily accessible to the thiol reagent. This is in sharp contrast to the C130H, D153T, and D153H variants of AK_{sub}, which reacted rapidly with DTNB (Figure 2A). The kinetics of the reaction with DTNB of these mutants at 20 °C and pH 7.4 was satisfactorily fitted to a single-exponential equation, except an initial missing amplitude which corresponds to the reaction of zinc-free enzyme already present at t_o of the experiments. Over 0.5 mM DTNB, the values of k_{obs} (1.3×10^{-2} s^{-1} for the C130H mutant, 0.8×10^{-2} s^{-1} for the D153T mutant, and 1.7×10^{-3} s^{-1} for the D153H mutant) were practically independent of the concentration of thiol reagent. Thus, the first-order process might reflect the dissociation rate of the AK–Zn^{2+} complex according to the simplest model:

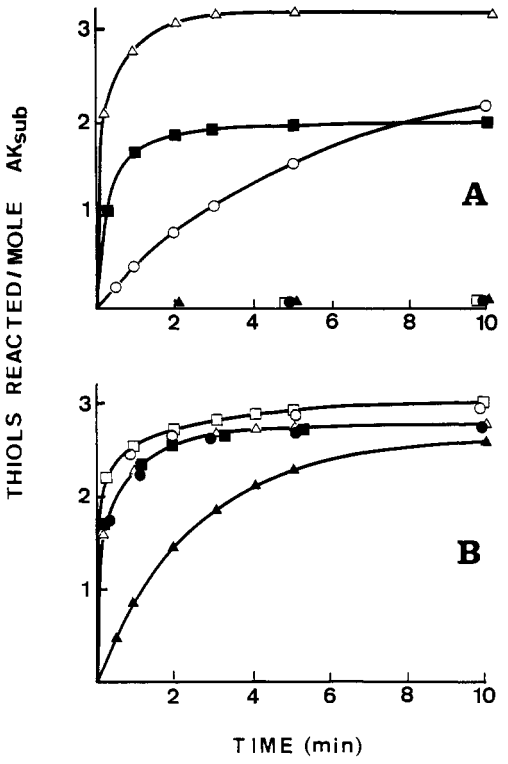
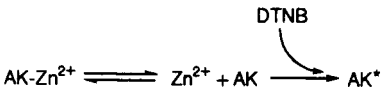


FIGURE 2: Reaction of AK_{sub} with DTNB under native conditions (A) or after removal of zinc (B). AK_{sub} (42 μM in 10 mM Tris-HCl, pH 8) was treated with 20 μL of 10 mM DTNB; then the absorbance increase was read at 412 nm (ϵ of thionitrobenzoate anion = 13.6 mM) up to 40 min after addition of reagent. The ratio of thiols reacted to moles of AK_{sub} was calculated using a molecular mass of 24 kDa. (●) Wild-type AK_{sub}, (□) H138N mutant, (▲) D153C mutant, (■) C130H mutant, (△) D153T mutant, and (○) D153H mutant.



Preincubation of C130H, D153T, and D153H mutants for 24 h with $ZnCl_2$ (between 10 and 500 μM) did not affect the k_{obs} values. One might assume that differences in affinity (K_d) for metal ion are primarily due to the dissociation rate constants for protein–metal complexes in various mutants. Under identical experimental conditions the k_{obs} values for the wild-type enzyme and the D153C and H138N mutants were 3.3×10^{-5}, 3.6×10^{-5}, and 2.8×10^{-5} s^{-1}, respectively.

As expected, the wild-type apo-AK_{sub} (metal-free protein) reacted readily with DTNB (~3 SH groups/mole of enzyme). The Zn^{2+}-free D153C mutant, on the other hand, was considerably less reactive (2.6 mol of SH reacted only after 40 min of incubation with DTNB) (Figure 2B). This reduced reactivity of the apo-D153C enzyme may be due to the formation of intramolecular disulfide bridges, a tendency favored by a configuration containing four free Cys residues.

Titration of AK_{sub} with PMPS, a strongly dissociating sulfhydryl reagent (Giedroc *et al.*, 1986; Hunt *et al.*, 1984), indicated a linear incorporation of up to 2 (C130H mutant), 3 (wild type and D153H, D153T, and H138N mutants), or 4 equiv of PMPS (D153C mutant) (Figure 3A). Zinc released during the reaction of AK_{sub} with PMPS was titrated with the metal-binding dye 4-(2-pyridylazo)resorcinol (PAR) (Hunt *et al.*, 1984). While the molar ratio of metal to protein was ≥0.8 for the wild-type enzyme and the D153C, H138N, and D153H mutants, it was much lower in the case of the C130H mutant (Figure 3B). In the course of these experiments we

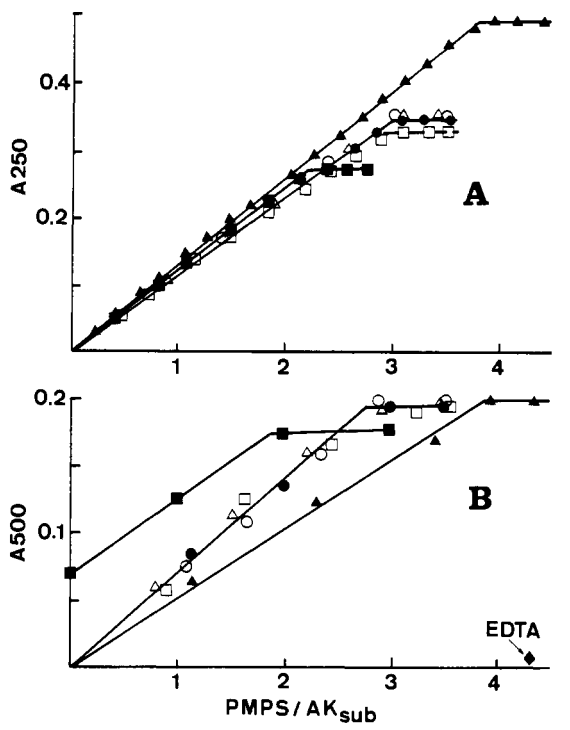


FIGURE 3: Titration of AK_{sub} with PMPS. (A) AK_{sub} (27 nmol in 0.6 mL of 10 mM Tris-HCl, pH 8) was treated with PMPS (1 mM solution in the same buffer) to give the indicated molar ratios of PMPS to enzyme. The absorption at 250 nm was measured relative to the control value at the beginning of the titration. (B) Titration was also performed in the presence of PAR, where the absorption developing at 500 nm reflects formation of a zinc–dye complex. Symbols are the same as those used in Figure 2. (◆) Indicates absorbance after addition of 2 mM EDTA.

found a relatively simple way of preparing metal-free AK_{sub}. Treatment of the native protein with an excess of PMPS and EDTA and subsequent desalting on a Sephadex G-25 column resulted in an enzyme that lost more than 90% of its Zn^{2+}. Addition of DTT (2 mM) almost completely restored the activity of each variant.

Stability of Wild-Type AK_{sub} and Mutant Proteins. In preliminary experiments *E. coli* extracts expressing various forms of AK_{sub} were heated for 10 min at temperatures between 30 and 65 °C, after which the residual adenylate kinase activity was determined. While wild-type AK_{sub} and the D153H, D153C, and H138N mutants were half-inactivated at about 52 °C, two mutants (D153T and C130H) showed a much lower thermal stability (half-inactivation occurred at ~40 °C).

The excess heat capacity curve for the wild-type AK_{sub} and those of three mutants are compared in Figure 4. The calorimetric curves were reversible (as inferred from their reproducibility in the second heating cycle), and each of these curves could be approximated by a two-state transition model. It is evident from Figure 4 that while the thermal denaturation profile of the wild-type protein is very similar to those of the D153C and H138N mutants, the stability of the C130H variant is severely reduced. This is reflected by both the lowering of the midpoint denaturation temperature (by almost 10 °C) and the decrease in the enthalpy of unfolding. The decrease in thermal stability of C130H mutant was not due to the formation of disulfide bonds. Determination of thiols with DTNB before and after heating of the protein at 50 °C (the temperature at which the C130H mutant was completely unfolded) gave identical results. The mean calorimetric denaturation parameters derived from two or three experiments

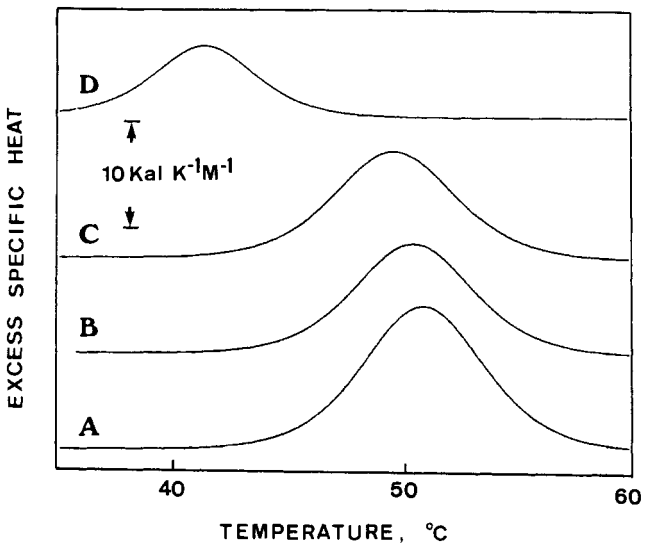


FIGURE 4: Differential scanning calorimetric traces for wild-type AK_{sub} (A), D153C mutant (B), H138N mutant (C), and C130H mutant (D). The curves shown here were obtained by smoothing the raw calorimetric data and subtracting from them the base lines by use of a cubic interpolation procedure.

Table 1: Calorimetric Parameters for the Denaturation of Wild-Type AK_{sub} and Three Mutants

enzyme	T_m[a] (°C)	ΔH_{cal}[b] (kcal/mol)	$\Delta\Delta G°$[c] (kcal/mol)
wild-type AK_{sub}	50.7	90	0
D153C mutant	50.4	70	0.06
H138N mutant	49.5	69	0.26
C130H mutant	41.3	38	1.10

[a] T_m is the temperature at which the denaturation of the protein is half-completed. [b] ΔH_{cal} is the calorimetric enthalpy of denaturation calculated from the area under the denaturational curve. [c] $\Delta\Delta G°$ is the standard free energy of denaturation corrected to the T_m of the wild-type protein, calculated using the Gibbs–Helmholtz equation, assuming the denaturation enthalpy to be independent of temperature.

with each protein are listed in Table 1. Data in the last column of this table represent the standard free energy of denaturation calculated at the T_m for the wild-type protein, i.e., 50.7 °C. The latter parameter may be interpreted as a standard free energy of destabilization of each mutant protein relative to the wild-type protein (Hecht *et al.*, 1984).

The reduced thermodynamic stability of the C130H mutant protein was corroborated by the equilibrium unfolding experiments in guanidine-HCl. Like in the case of thermal denaturation, the chemical unfolding of all proteins studied (Figure 5) can be approximated by a two-state transition model. The guanidine-HCl unfolding profiles for the H138N and D153C mutants (data not shown) are very similar to that for the wild-type protein. This may be contrasted with the much lower stability of the C130H variant. The midpoint transition concentration of guanidine-HCl for this protein is 0.53 M, compared to 1.38 M for the wild-type enzyme.

The stability of the various AK_{sub} mutants, as determined by DSC and equilibrium unfolding experiments, correlated reasonably well with the susceptibility of these proteins to proteolytic digestion. As shown in Table 2, under identical experimental conditions the wild-type enzyme and the D153H variant exhibit similar resistance to inactivation by trypsin, while the rates of proteolytic inactivation for the C130H and D153T mutants are 10 and 7 times higher than that of the wild-type enzyme. The D153C and H138N variants show the highest resistance to trypsin inactivation of all of the

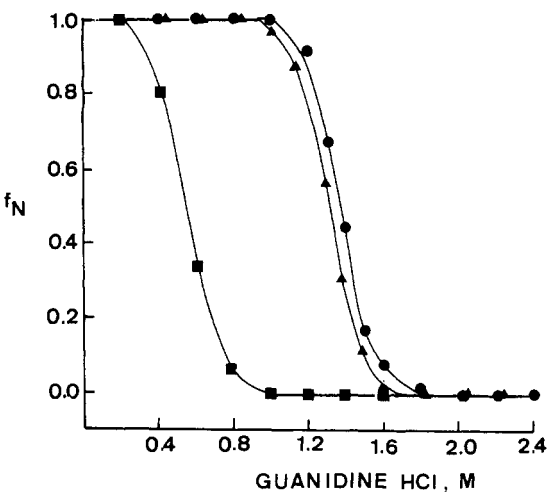


FIGURE 5: Dependence of the apparent fraction of folded proteins, f_N, on the guanidine-HCl concentration for the wild-type AK_{sub} (●) and the H138N (▲) and C130H mutants (■).

Table 2: Proteolysis by Trypsin of Wild-Type AK_{sub} and of Various Mutants Obtained by Site-Directed Mutagenesis[a]

adenylate kinase	$k \times 10^4$ (s^{-1})
B. subtilis, wild type	11.1
B. subtilis, D153C	7.9
B. subtilis, H138N	8.9
B. subtilis, D153H	12.0
B. subtilis, D153T	78
B. subtilis, C130H	106
E. coli, wild type	102
B. stearothermophilus, wild type	1.1

[a] Enzymes at 1 mg/mL in 50 mM Tris-HCl (pH 7.4) were incubated at 30 °C with TPCK-trypsin (20 μg/mL). Aliquots of 20 μL were withdrawn at different time intervals and supplemented with 3 μg of soybean trypsin inhibitor. Residual activity was measured in the direction of ATP formation. Inactivation with trypsin was first-order for about 90% activity loss; rate constants (k) were determined from the slopes of semilog plots of enzyme activity versus time. Controls run in the absence of trypsin showed no inactivation of various enzymes during incubation. For comparison, the sensitivities to trypsin inactivation of *E. coli* and *B. stearothermophilus* adenylate kinase are also shown.

proteins tested. This is, however, not accompanied by an increased thermodynamic stability of these proteins (see above) and likely reflects a small local conformational change in the vicinity of one of the potential cleavage sites.

Spectroscopic Studies. In the search for structural differences between different variants of AK_{sub}, we turned to molecular spectroscopic methods.

(A) Infrared Difference Spectra. Illustrated in Figure 6 are two difference spectra obtained by subtracting the absorbance spectra of the mutants C130H and D153C from the absorbance spectrum of wild-type AK_{sub}. It is well established that spectral features in the 1600–1700 cm^{-1} (amide I) region of the infrared spectra of proteins are characteristic of their secondary structure (Surewicz *et al.*, 1993), while the 1500–1600 cm^{-1} region contains absorption bands that originate from side vibrations (Chirgadze *et al.*, 1975). An essentially flat infrared difference spectrum such as that shown in trace B of Figure 6 (D153C mutant) illustrates that substitution of Cys for Asp in position 153 has only a minor effect on the overall structure of AK_{sub}. On the other hand, there are major differences between the spectra of the wild-type AK_{sub} and its C130H mutant. Positive bands in spectrum A of Figure 6 represent structural features present in the wild-type protein that are not found in the mutant; negative bands represent structural features found in the

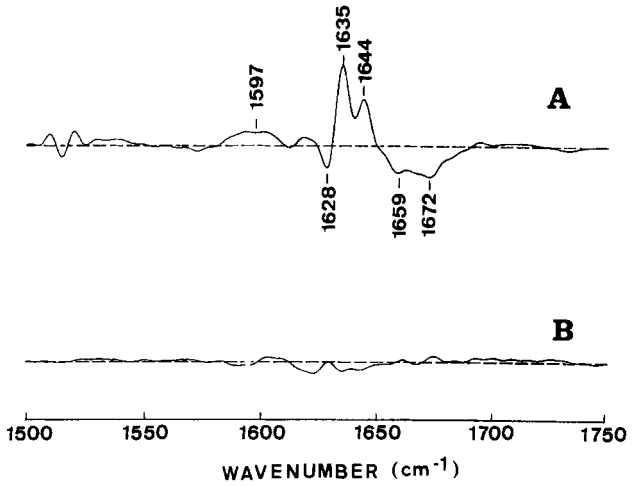


FIGURE 6: Infrared difference spectra in the 1500–1750-cm^{-1} region. The spectra were obtained by subtracting the absorbance spectrum of the C130H mutant (trace A) or that of the D153C mutant (trace B) from the absorbance spectrum of wild-type AK_{sub}. The three absorbance spectra were measured under strictly comparative conditions.

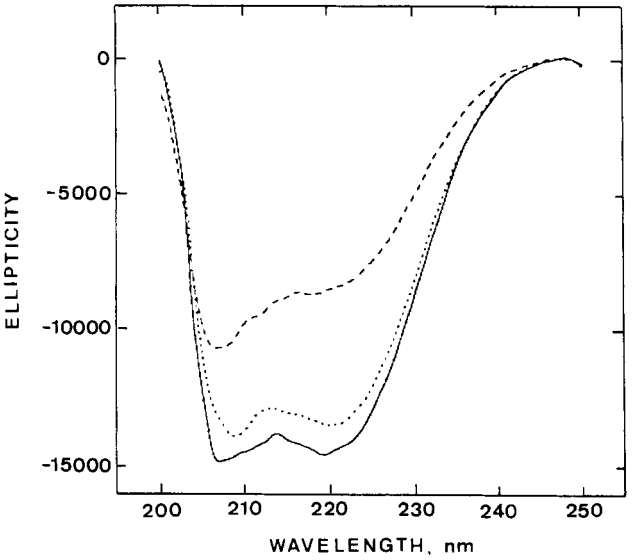


FIGURE 7: Far-UV circular dichroism spectra of wild-type AK_{sub} (—), D153C mutant (- - -), and C130H mutant (– –).

mutant but not in the wild-type protein. While there are recognized difficulties with the quantitative determination of the secondary structures of proteins from their infrared spectra (Surewicz *et al.*, 1993), certain conclusions can be drawn from these infrared difference spectra. Thus, the positive bands at 1635 and 1644 cm^{-1} together with the negative bands at 1659 and 1672 cm^{-1} are indicative of more secondary structure in the wild-type AK_{sub} and suggest that certain features due to β-type structures (band at 1635 cm^{-1}) and α-helical structures (band at 1644 cm^{-1}) in the wild-type protein are replaced by more irregular structures (band at 1659 cm^{-1}) and turn-containing structures (band at 1672 cm^{-1}) in the C130H mutant. The features around 1517 cm^{-1} are indicative of a change in the environment of one or more tyrosine residues, which have a characteristic absorption band at this frequency.

(B) CD Spectra. The effect of single amino acid substitutions on the secondary structure of AK_{sub} was confirmed by CD spectroscopy (Figure 7). The far-UV CD spectrum of AK_{sub}, characterized by negative minima at 222 and 208 nm, indicates a high content of α-helical structure. While the

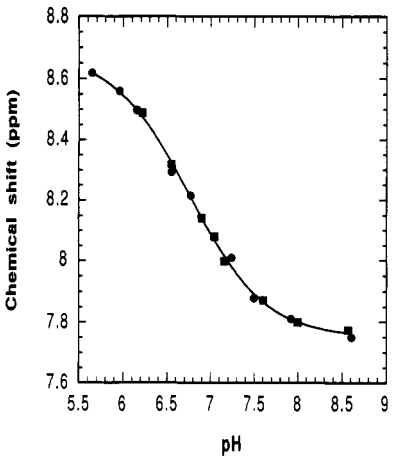


FIGURE 8: NMR titration curve for His C_2 proton resonances in wild-type AK_{sub}. Protein samples were approximately 0.2 mM in phosphate buffer (100 mM) at 310 K. Circles and squares represent two different enzyme preparations.

spectrum of the D153C mutant was identical with that of the wild-type protein, the largest difference in the spectral intensity was found again with the C130H mutant, suggesting an alteration of the overall protein conformation which would also account for the unusual reactivity of the C130H mutant with DTNB.

(C) NMR Spectra. A comparative analysis of the aromatic region in the NMR spectra of the wild-type protein and the H138N and D153C mutants shows that a narrow line at low field is highly sensitive to pH variation between pH 5.50 and 8.60. This resonance most likely corresponds to a C_2H proton of a histidine side chain (Markley, 1975). Because the peak is also observed in the H138N variant, it may be assigned to the C_2 proton of His28. The pH dependence of this resonance (Figure 8) was identical for three investigated variants of AK_{sub} (pK_a = 6.76) and close to that observed for a free histidine (pK_a = 6.60) (Markley, 1975). These results suggest that in these three types of AK_{sub} His28 is accessible to the solvent and is not involved in ionic interactions with charged or polar neighboring side chains. The C_4 proton resonance can be tentatively assigned to a narrow signal at 6.80 ppm (at pH 6.55) which has a weak pH dependence (less than 0.20 ppm) and was observed in all AK variants studied here.

Comparison of the aromatic region of the wild-type and H138N variants showed a number (at least six) of spectral differences which are reproducible throughout the studied pH range. This kind of spectral change may be caused by conformational rearrangements, at least at the level of aromatic side chains, induced by the His to Asn substitution. More significant for our study was the observation of a singlet resonance at 7.38 ppm (pH insensitive), which is absent only in the spectrum of H138N. It may be a candidate for the C_2 proton resonance of the second His residue (H138). Whatever the correct assignment may be, this His residue shows an abnormal chemical shift and pH dependence. Such a behavior is usually associated with the presence of a metal ligand or the close proximity of a positively charged side chain.

Spectral changes in the aromatic region were also observed for the D153C variant. At basic pH the C_2H resonance of His28 is accompanied by three titratable peaks of similar line width but lower intensity. A simple explanation would be the formation of intra- or intermolecular disulfide cross bridges inducing a molecular heterogeneity. No other pH-dependent spectral changes were observed in the aromatic region of the NMR spectrum of the D153C mutant.

DISCUSSION

Zinc is frequently encountered in proteins and may play either a catalytic role, a structural role, or both (so-called cocatalytic zinc). While catalytic zinc participates directly in the forming or breaking of chemical bonds, the principal role of structural zinc is to stabilize the native tertiary structure of the protein. *B. stearothermophilus* AK has been shown recently to coordinate zinc through four Cys residues (Cys130, Cys133, Cys150, and Cys153) which form a zinc-finger-like motif (Glaser *et al.*, 1992). Removal of the metal atom from AK_{st} results in a lowering of the denaturation temperature of the protein by 7 °C, although it does not affect the catalytic activity in the presence of reducing agents. AK from *B. subtilis*, highly homologous with AK_{st}, lacks the fourth Cys at position 153, which is replaced by an Asp. Since the carboxylate oxygens are known to primarily coordinate catalytic zinc atoms, we first hypothesized that one of the two His residues in AK_{sub} (His28 or His138) may act as the fourth partner in the metal chelation. Both amino acids are conserved in the zinc-chelating forms of AK, which are all obtained from Gram-positive bacteria (Gilles *et al.*, 1994). Moreover, His28 is replaced by a Gln residue in the enzyme from Gram-negative organisms such as *E. coli* which are devoid of zinc. The C_2 proton of His28, sensitive to pH variations and much like the C_2H in AK1, was readily assigned in the NMR spectrum of AK_{sub}. Being accessible to the solvent and uninvolved in interactions with charged or polar side chains, His28 is not a likely candidate for chelating zinc in AK_{sub}. On the contrary, the unusual high-field shift (7.38 ppm) and the pH independence of the C_2 peak of His138 in AK_{sub} argue for a potential role of this amino acid in binding the metal atom. However, an equivalent His residue in AK_e (His134) has been shown to behave similarly (C_2H resonance at 7.50 ppm) (Bock *et al.*, 1988). This effect is probably due to the proximity (less than 0.4 nm) of Phe137, which is conserved as Phe141 in AK_{sub}. In order to eliminate any ambiguity concerning the role of His138 in zinc chelation in AK_{sub}, we have substituted it with Asn. This sterically conservative replacement residue with a similar polarity had no significant effect on the structure, stability, or zinc-binding properties of AK_{sub}. Thus, the present data rule out the likelihood that His residues participate in zinc coordination in wild-type AK_{sub}. Another substitution made in this study, that of Cys for Asp153, had practically no effect on the structural or catalytic properties of AK_{sub}, with the exception of conferring upon the protein a slightly higher resistance to inactivation by trypsin. Furthermore, the observation that Cys153 in the AK_{sub} mutant was not accessible to DTNB provides an indication that a "four-Cys" configuration for metal chelation might have been acquired by the mutant enzyme (Figure 9). Although the central question of the geometry of zinc coordination in wild-type AK_{sub} still remains to be answered, our data have opened for the first time the possibility that three thiols and a carboxylate suffice for a tight anchoring of the zinc in AK_{sub}. This possibility appears rather intriguing within the framework of current models pertaining to the chelation of structural zinc atoms (Vallee & Auld, 1990, 1993). However, substitution of Asp153 with a Thr residue and to a lesser extent with a His residue yielded variants of bacterial enzymes that lost the metal with concomitant inactivation.

Of all the mutations investigated herein, those which proved to have a most profound effect on the structure and stability of the enzyme were the substitutions of Cys130 with His and of Asp153 with Thr. The C130H variant appeared to have a severely reduced content of ordered secondary structure,

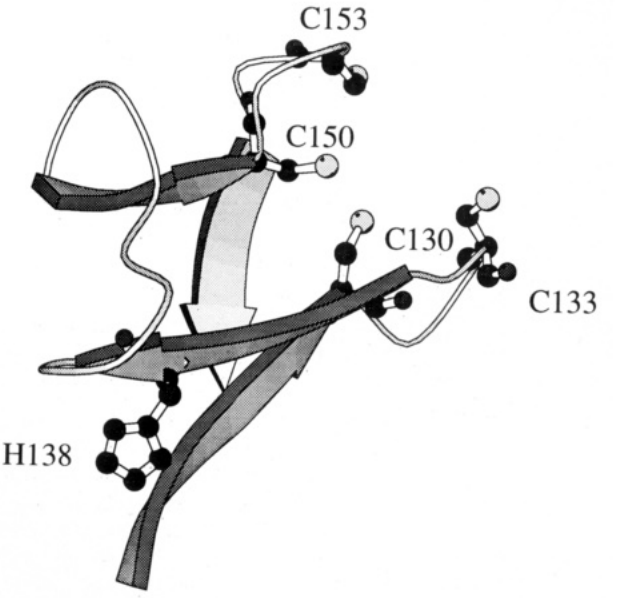


FIGURE 9: Pictorial representation of the three-dimensional structure of the zinc-binding domain in AK_{sub}. The model was obtained by starting from the atomic coordinates of AK_e (Müller & Schulz, 1992) and substituting four Cys side chains (as in the D153C mutant) in the homologous positions. No search for side-chain optimization was made after substitution. The figure was obtained with the MOLSCRIPT software (Kraulis, 1991).

and its thermodynamic stability was drastically reduced. These data point to a critical role of Cys130 in maintaining the native three-dimensional structure of AK_{sub}. In fact, substitution of Cys130 with histidine in AK_{st} had exactly the same effect on the protein molecule, i.e., a decreased thermodynamic stability and a severely reduced ability to bind zinc (V. Perrier *et al.*, in preparation). The same conclusions seem to apply to Asp → Thr substitution.

Inactivation of the C130H and D153T mutants in the absence of reducing agents is due to the formation of disulfide bridges between the remaining and now accessible Cys residues. This interpretation is supported by the following observations: (i) SDS–PAGE of the C130H and D153T mutants in the presence or absence of reducing agents yielded single protein bands of apparent molecular mass of 27.5 kDa. (ii) The air-exposed C130H mutant did not react with PMPS or with DTNB to a significant extent. However, in the absence of high-resolution structural data, it is difficult to explain how these new steric constraints imposed upon the AK_{sub} molecule can lead to a loss of its catalytic activity. With respect to the zinc-binding properties, it is worthwhile noting that His at position 130 is found in AKs from Gram-negative bacteria such as *E. coli* or *Paracoccus denitrificans*, all of which are devoid of structural metal ions. The Cys130 → His substitution in AK_{sub} (as well as the Asp153 → Thr substitution) decreased the affinity of the protein for zinc considerably, although the interaction between the metal ion and the enzyme was not completely lost. Thus, after SDS–PAGE and electrotransfer onto nitrocellulose, the C130H mutant incorporated radioactivity when sheets were incubated with $^{65}ZnCl_2$. This suggests that the metal-binding property of zinc-containing AKs has been acquired through several mutational events and that the coordination pattern of the metal ion rather allows for a species-specific variability. This hypothesis is currently under investigation in our laboratory.

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