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Structural and Catalytic Properties of a Deletion Derivative ($\Delta_{133-157}$) of *Escherichia coli* Adenylate Kinase*

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Escherichia coli **adenylate kinase (AKe) as well as the enzyme from yeast and mitochondria differs from the muscle cytosolic variant (AK1) by an insertion of 25 amino acid residues that are missing in AK1. The extra sequence, highly homologous in "large" size variants, is situated between residues 133 and 157 in AKe. Removal of 25 codons in the corresponding *adk* gene resulted in expression of a modified form of adenylate kinase ($\Delta_{133-157}$ AKe) which still conserved 7% of the maximal activity of the wild-type protein. The apparent K_m for nucleotide substrates was increased by a factor of 4.6 (ADP), 23 (ATP) or 43 (AMP) in $\Delta_{133-157}$ AKe when compared with the wild-type enzyme. The secondary structure of $\Delta_{133-157}$ AKe, as well as its thermal stability were very similar to the parent protein. However, the deleted protein was much more sensitive than the wild-type enzyme to inactivation by trypsin. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of trypsin digested $\Delta_{133-157}$ AKe revealed accumulation of several well defined fragments which were not observed in the case of wild-type enzyme. We conclude that the additional sequence, although necessary for expression of full activity in AKe, is not critical for catalysis. It is perhaps responsible for interaction of enzyme with other cellular components although a different mechanism of water shielding for large and small size variants of AK can be also envisaged.**

Adenylate kinase (AK, ATP:AMP phosphotransferase, EC 2.7.4.3),[1] which catalyzes the reaction,

$$ATP \cdot Mg^{2+} + AMP \Leftrightarrow ADP \cdot Mg^{2+} + ADP,$$

is an attractive model enzyme for studying structure-function relationships, as it is a small monomeric protein, easy to purify, handle, and detect (1, 2). The alignment of primary structures of different isoforms from different organisms (3) showed a striking difference between mammalian muscle cytosolic adenylate kinase (AK1) and the enzyme from *Saccharomyces cerevisiae* (AKy), *Escherichia coli* (AKe) or mammalian mitochondria (AK2 and AK3). The latter four "long AKs" contain a continuous insertion of 25 amino acids that are missing in the short variant (AK1) of adenylate kinase. This insertion is bracketed by identical residues in all species of adenylate kinase: Ser-Gly-Arg at the N terminus and Asp-Asp at the C terminus (see Figs. 1 and 2). The three-dimensional structures of long and short adenylate kinases are remarkably similar (4–7). A schematic view of the structure of the long AKs as inferred from the three-dimensional analysis of the yeast and *E. coli* adenylate kinase complex with the bisubstrate analog Ap_5A (4, 6) is shown in Fig. 1. Although the extra sequence is not as well defined as the rest of the structure it was found to be located close to the ATP binding site (adenine A of Ap_5A). It seems to act as a lid that shields the active site from the surrounding medium. Additionally, NMR experiments suggest that His^{134} of AKe (equivalent to His^{143} in AKy) is located close to the $ATP \cdot Mg^{2+}$ binding site (8, 9).

Since the extra sequence is highly conserved in long AKs (13 amino acid residues from 25 are identical in AK2, AK3, AKy, and AKe), we wanted to discover its possible functional role in this enzyme. We deleted the whole sequence situated between residues 133–157 in AKe and investigated the structural and catalytic properties of the modified enzyme.

EXPERIMENTAL PROCEDURES

Chemicals—Adenine nucleotides, restriction enzymes, T4 DNA ligase, and coupling enzymes were from Boehringer Mannheim. DNA polymerase large fragment (Klenow) was from Du Pont-New England Nuclear. TPCK-treated trypsin and soybean trypsin inhibitor were from Sigma. Blue-Sepharose, polybuffer exchanger 94, and polybuffer 74 were from Pharmacia LKB Biotechnology Inc. Oligonucleotides were synthesized according to the phosphoamidinate method using a commercial DNA synthesizer (Cyclone™ Biosearch).

Bacterial Strains, Plasmids, and Cloning Methods—Plasmid pEAK91, which contains the *adk* gene from *E. coli* on the high copy number plasmid pEMBL9 has been described previously (10). AKe is expressed from this plasmid in strain CK600 (SupE, $hsdM^+$, hsdh), which contains a chromosomal copy of the *adk* wild-type gene. For the construction of the deleted gene involving *Bcl*I restriction enzyme we used strain GM119Δ which is dam^- with unknown genetic background. Thermosensitive strains CV2 and KG2 are *plsA2* (=*adk2*) *glpD3, glpR2Δ phoA8 tonA22* T2-R *rel1A1* (λ)*pit-10 om* pF627 *fad-*

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[1] The abbreviations used are: AK, adenylate kinase; Ap_5A, P^1,P^5-di(adenosine 5′)-pentaphosphate; $\Delta_{133-157}$ AKe, deletion derivative of *E. coli* AK lacking amino acid residues from 133 to 157; blue-Sepharose, Cibacron blue 3G-A-Sepharose CL-6B; HPLC, high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TPCK, L-1-tosylamido-2-phenylethylchloromethyl ketone; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

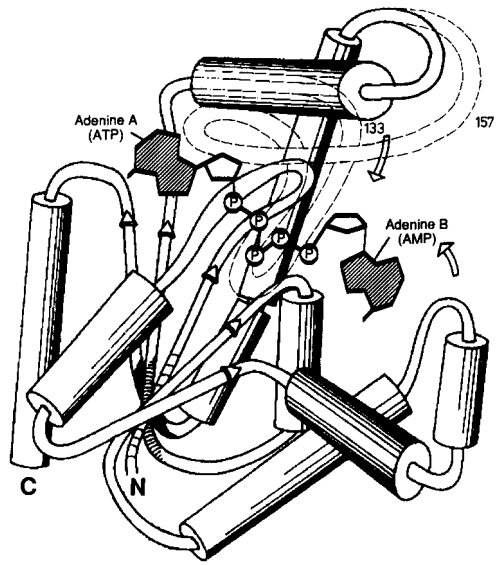

FIG. 1. **Schematic drawing of the three-dimensional structure of the Ap_5A complex of AKe based on the model of AK1, a short version of adenylate kinase.** The location of the extra sequence is tentative and was inferred from the structure of the AKy·Ap_5A complex (4). The *two arrows* indicate the movement of the structural elements involved in the induced fit mechanism (see "Discussion").

L701, and *plsA15* (=*adk15*) recA1 proC24 thyA25 nalA12 argG34 metB1 pyrC30 lac str-97 tsx-63 mlt-2 xyl-7 or 14, respectively. They were transformed with pEAK91 ($\Delta_{133-157}$) and plated at 28 °C. After 48-h of growth, 100 colonies from each transformation were replated at 28 and 42 °C and scored for survival after 24 h (42 °C plates) and 48 h (28 °C plates). Mutagenesis was done using the thiophosphate method of Taylor *et al.* (11). If not indicated otherwise, the cloning techniques were performed as described by Maniatis *et al.* (12). Sequencing of the construction was performed with the T7 polymerase sequencing kit from U. S. Biochemical Corp.

Purification of Adenylate Kinase and Activity Assays—The wild-type enzyme from AKe-overproducting strain of *E. coli* was purified as described previously (13). $\Delta_{133-157}$ AKe was purified essentially by the same procedure involving chromatography on blue-Sepharose and Ultrogel AcA54. Enzyme fixed on a blue-Sepharose matrix at pH 7.4 was eluted either with a salt gradient (between 0 and 1 M NaCl in 50 mM Tris-HCl, pH 7.4) or with a mixture of 3 mM ATP + 3 mM AMP in the same buffer. AKe expressed by the chromosomal wild-type *adk* gene coeluted with $\Delta_{133-157}$ AKe both in blue-Sepharose and Ultrogel AcA54 chromatography. The two proteins were completely separated by chromatofocusing allowing an accurate structural and kinetic analysis of the modified protein (see Fig. 4, under "Results"). Adenylate kinase activity was determined with the coupled spectrophotometric assay (13) at 334 nm and 30 °C in 0.5-ml final volume on a Eppendorf PCP6121 photometer. One unit of enzyme activity corresponds to 1 μmol of product formed in 1 min at 30 °C and pH 7.4.

Trypsin Digestion and Peptide Separation of Deleted AKe—Wild-type and deleted forms of AKe were digested for 15 h at 37 °C with TPCK-trypsin (1%, w/w). Peptides were purified by reverse-phase HPLC on a Perkin-Elmer apparatus (series 410 equipped with a LC135 diode array detector) using a Nucleosil C-18 column (5 μM, 4.6 × 25 mm) and an ammonium acetate, pH 6.0/acetonitrile elution system at a flow rate of 1 ml/min and absorbance recording at 230 and 280 nm.

Amino Acid and Sequence Analysis—Amino acid analyses were performed on a Beckman System 6300 high performance analyzer after 6 N HCl hydrolysis for 22 h at 110 °C. Manual sequencing of the isolated peptide was conducted using the 4-(dimethylamino)azobenzene 4′-isothiocyanate/phenylisothiocyanate double coupling technique (14).

Spectroscopic Studies—Infrared spectra were recorded with a Digilab FTS-60 spectrometer using a high sensitivity deuterated tryglicine sulfate detector. Samples were prepared in 50 mM HEPES buffer in D_2O, pH 7.4, at a protein concentration of 1 mM, and the spectra were obtained as described previously (15). Circular dichroism (CD) spectra were recorded on a Jobin-Yvon CD6 or a Jasco-600 apparatus from 180 to 260 nm using quartz cylindrical cells of 0.2 or 0.5 mm and protein concentration ranging from 2 to 40 μM. Results are given as the mean residue molar ellipticity (θ) expressed in degrees·cm^2·dmol^{-1}. For the estimation of secondary structure, CD curves in the 190–260 nm range were analyzed by the method of Chen *et al.* (16) using the program of Yang *et al.* (17). The equilibrium unfolding as a function of guanidine HCl was monitored at 20 °C and 222 nm. All samples were equilibrated for approximately 12 h before measurements were made. 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.

Thermal Denaturation—The thermal stability of the wild-type AKe and the $\Delta_{133-157}$ AKe was studied by high-sensitivity differential scanning calorimetry using a Microcal MC-2D instrument at a scanning rate of approximately 50 °C/h. Protein solutions used in these experiments were prepared in 50 mM HEPES buffer, pH 7.4, at a concentration of 2–2.5 mg/ml.

RESULTS

Expression and Purification of $\Delta_{133-157}$ AKe, a Deleted Derivative of AKe—The *E. coli adk* gene on the high expression plasmid pEAK91 was mutated such that Asp-Ile substituted for Tyr^{133}-His^{134}, thus creating an *Eco*RV restriction site (Fig. 2). Bacteria expressing the doubly-modified enzyme (Y133D, H134I) exhibited an AK activity close to that of the same *E. coli* strain without plasmid (about 1 unit/mg of protein). The modified pEAK91 plasmid was digested with *Eco*RV and *Bcl*I. The *Bcl*I site was filled in with Klenow polymerase, the 75-base pair fragment removed and the plasmid religated. The resulting clones were screened for the presence of *Bcl*I site and the absence of the *Eco*RV site, and a positive clone was sequenced. The sequence of the resulting plasmid around the

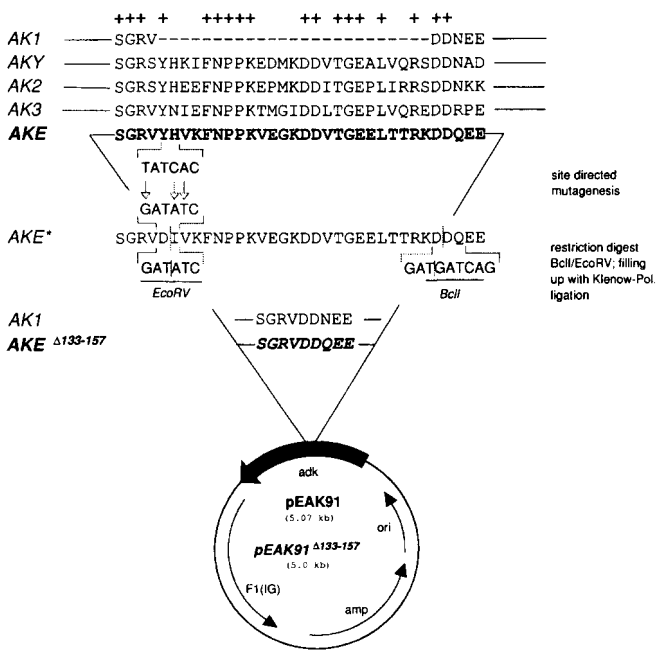

FIG. 2. **Construction of the expression plasmid for $\Delta_{133-157}$ AKe.** The sequence alignment of short and long AKs (3) shows the conserved residues in and around the extra sequence. Three point mutations were introduced into single-stranded DNA from plasmid pEAK91 (10) to create an *Eco*RV site at the N-terminal site of the deletion. The *Bcl*I site was cleaved, filled in using Klenow polymerase, and after digestion with *Eco*RV the 75-base pair fragment was removed, the plasmid religated and analyzed, as described under "Experimental Procedures." The resulting amino acid sequence of $\Delta_{133-157}$ AKe is shown in comparison with the sequence of AK1 (pig muscle).

deletion site was now highly homologous to the sequence of mammalian cytosolic AK (Fig. 2). The soluble extract from *E. coli* expressing plasmid pEAK91 ($\Delta_{133-157}$) exhibited a 2-fold higher specific activity under standard assay conditions (30 °C, pH 7.4, and 1 mM ADP as substrate) as compared with the same *E. coli* strain without plasmid. As several temperature sensitive mutants of the *adk* gene have been described showing that it is an essential gene, we wanted to know whether adk^{ts} strains could be complemented by the deleted *adk* gene. Two types of temperature sensitive mutants (CV2 with a P87S mutation and KG2 with a S129F mutation) (3) were transformed with plasmid pEAK91 ($\Delta_{133-157}$). The colonies were viable at permissive and nonpermissive temperatures, showing that the deletion derivative is not only active, but that the deleted region is not important for the viability of *E. coli*.

$\Delta_{133-157}$ AKe was expressed in a high yield in *E. coli* strain CK600 and purified by standard procedures involving blue-Sepharose and gel permeation chromatography (13). Under these conditions, the protein co-eluted with the wild-type AKe expressed by the chromosomal gene (Fig. 3). The two proteins were subsequently separated by chromatofocusing (Fig. 4) as the pI of deleted enzyme (5.4) was higher than that of the wild-type protein (5.1).

Structural Analysis of $\Delta_{133-157}$ AKe—Amino acid analysis of $\Delta_{133-157}$ AKe was in good agreement with what was expected from the removal of the sequence situated between Tyr^{133} and Lys^{157} in the wild-type protein (Table I). To confirm the extent of deletion, digests with TPCK-trypsin of both wild-type and deleted AKe were analyzed by reverse-phase HPLC. The elution profile of tryptic peptides indicated that the two peaks corresponding to peptides 132–136 and 137–156 in wild-type AKe were missing in the deleted enzyme (Fig. 5). A new tryptic peptide in $\Delta_{133-157}$ AKe arising from the fusion of Val^{132} to Asp^{158} (VDDQEETVR) had an elution time very similar to that of peptide ^{157}KDDQEETVR^{165} in the wild-type enzyme.

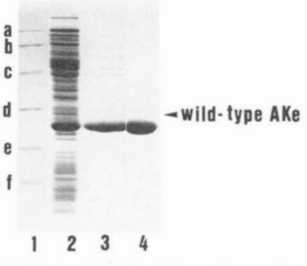

FIG. 3. **SDS-PAGE (12.5%) of fractions obtained during the purification of $\Delta_{133-157}$ AKe.** *Lane 1*, standard proteins, from *top* to *bottom*: *a*, phosphorylase *a* (94,000); *b*, bovine serum albumin (67,000); *c*, ovalbumin (43,000); *d*, carbonic anhydrase (30,000); *e*, soybean trypsin inhibitor (20,100); *f*, lysozyme (14,400). *Lane 2*, bacterial extract (35 μg of protein). *Lane 3*, blue-Sepharose chromatography (35 μg of protein). *Lane 4*, Ultrogel AcA54 chromatography (18 μg of protein).

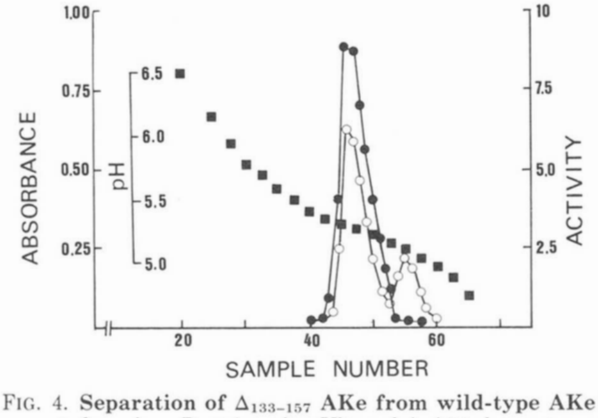

FIG. 4. **Separation of $\Delta_{133-157}$ AKe from wild-type AKe by chromatofocusing.** Proteins after Ultrogel AcA54 chromatography (5 mg) were loaded onto a polybuffer exchanger 94 column (0.5 × 15 cm). 15 ml of Tris-HCl 50 mM, pH 7.4, were used to wash the column. Proteins were then eluted with 35 ml of 10-fold diluted polybuffer 74 adjusted to pH 4 with 1 N HCl. Samples of 0.5 ml were collected at a flow rate of 4 ml/h. ■, pH; ●, absorbance at 280 nm; ○, activity (units/ml).

Figure 7 shows the excess heat capacity *versus* temperature curve for the wild-type AKe and the deleted protein. These curves were obtained by substracting the base lines from the raw calorimetric data using a cubic splines interpolation procedure. The thermodynamic parameters derived from the excess heat capacity curves (Table II) indicate relative close thermodynamic stabilities of both proteins. In particular, there is no significant difference between the denaturation temperatures of the wild-type AKe and its deletion derivative. Similar stabilities of the wild-type AK and the $\Delta_{133-157}$ proteins were also demonstrated by the results of equilibrium unfolding experiments in guanidine HCl (Fig. 8). For both proteins, the dependence of the folded fraction, f_N, on the denaturant concentration indicates a two-state unfolding process. The midpoint transition concentrations of guanidine HCl were 0.93 and 0.90 M for the AKe and $\Delta_{133-157}$ AKe, respectively (Fig. 8).

Kinetic Properties of $\Delta_{133-157}$ AKe—Table III shows the kinetic parameters of $\Delta_{133-157}$ AKe compared with AKe and rabbit muscle AK1. Deletion of the 25-amino acid extra sequence in *E. coli* protein decreased the catalytic activity by a factor of 62 (ATP formation) or 14 (ADP formation). The apparent K_m for nucleotide substrates was increased by a factor of 4.6 (ADP), 23 (ATP), or 43 (AMP). Under identical experimental conditions rabbit muscle AK1 exhibited kinetic parameters close to those of wild-type AKe. The requirements for divalent cation were practically the same for all three forms of adenylate kinase (not shown). However, excess AMP (4 mM) inhibited the wild-type AKe by about 50%, whereas a 2-fold higher concentration of nucleoside monophosphate did not inhibit the deleted bacterial variant.

DISCUSSION

The results of this study reveal numerous functional and structural similarities between $\Delta_{133-157}$ AKe and the wild-type protein. In particular both enzymes showed intrinsic transphosphorylating activities and similar resistance to tempera-

TABLE I

Amino acid composition of $\Delta_{133-157}$ adenylate kinase of E. coli compared with that of wild-type protein

Amino acid	Residues/molecule			
	$\Delta_{133-157}$		Wild-type	
	Observed	Calculated	Observed	Calculated[a]
Cys	ND[b]	1	ND[b]	1
Asx	16.7	18	19.9	21
Thr	6.8[c]	8	9.6[c]	11
Ser	4.0[c]	5	4.1[c]	5
Glx	22.1	23	25.0	26
Pro	8.0	8	10.3	10
Gly	17.2	18	19.6	20
Ala	19.0	19	19.0	19
Val	15.4	16	18.2	19
Met	ND[b]	6	ND[b]	6
Ile	12.5	14	12.3	14
Leu	15.5	15	16.1	16
Tyr	4.9[c]	6	5.9[c]	7
Phe	3.9	4	4.8	5
His	2.2	2	3.1	3
Lys	12.7	14	17.1	18
Arg	11.6	12	12.6	13

[a] From the nucleotide sequence of the *adk* gene (10).
[b] ND, not determined.
[c] Uncorrected values determined after 20 h of hydrolysis.

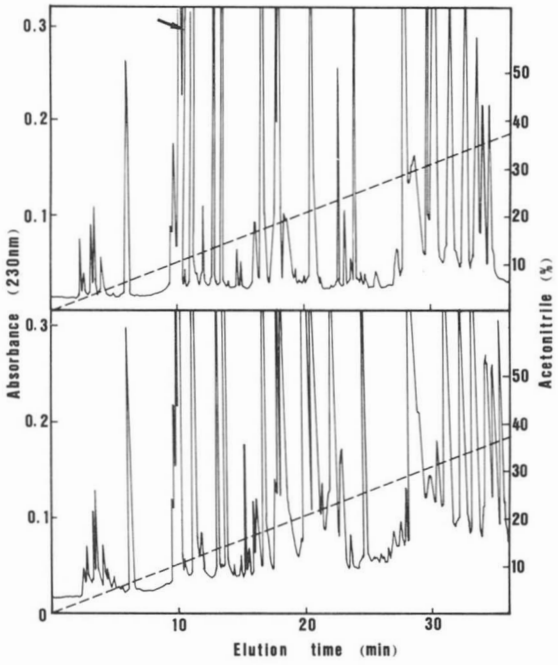

FIG. 5. **Separation of tryptic peptides by reverse-phase HPLC on a nucleosil C_{18} column.** Peptides resulting from 1 mg of digested protein were eluted with a linear gradient of 0–60% acetonitrile (- - - -) and detected by their absorbance at 230 nm (——). The flow rate was 1 ml/min. *Upper*, digest of $\Delta_{133-157}$ AKe. *Lower*, digest of wild-type enzyme. *Arrow* indicates the peptide resulting from the fusion of Val^{132} to Asp^{158}.

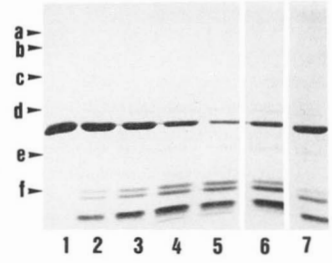

FIG. 6. **Proteolysis of $\Delta_{133-157}$ AKe by trypsin and protection by nucleotides.** $\Delta_{133-157}\Delta$ AKe at 1 mg/ml in 50 mM Tris-HCl, pH 7.4, and 50 mM KCl was incubated at 30 °C with TPCK-trypsin (2 μg/ml) in the absence (*lanes 1–5*) or presence of 4 mM ATP (*lane 6*) or 1 mM Ap_5A (*lane 7*). At different time intervals (0 min, *lane 1*; 1 min, *lane 2*; 2 min, *lane 3*; 5 min, *lane 4*; and 10 min, *lanes 5–7*), 20-μl aliquots were withdrawn, boiled with electrophoresis buffer, and analyzed by SDS-PAGE (12.5%) and Coomassie Blue staining. The molecular weight standards are the same as indicated in Fig. 3.

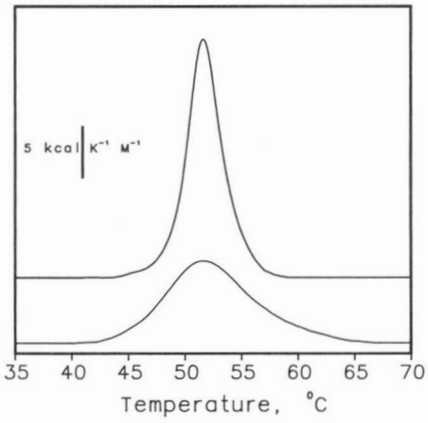

FIG. 7. **Differential scanning calorimetry curves of excess specific heat *versus* temperature for the wild-type AK (*upper trace*) and the $\Delta_{133-157}$ enzyme (*lower trace*).**

ture and guanidinium HCl denaturation. This suggests that the 25-amino acid residue insertion present in long variants of AKs is not essential for catalysis or for the maintenance of thermodynamically stable structures.

Despite these similarities, there are significant differences between AKe and $\Delta_{133-157}$ AKe. First, the deletion of the extra sequence reduced the catalytic activity of AKe drastically with a concomitant large increase of the K_m values for nucleotide substrates. The crystal structure analysis of AK1 without substrate of AK3 with AMP and of AKe and AKy complexed to the bisubstrate inhibitor Ap_5A has been completed (4, 6, 19). It revealed that the two adenine moieties of Ap_5A are located in the AMP and ATP binding sites and that the enzyme undergoes a stepwise transition from an open config-

uration, as shown in AK1, to a closed more compact structure when both substrates are bound. This structural change leads to an induced fit of the substrates (20). In kinases it also served to shield the active site from the surrounding water (21). The structural regions involved in this movement of two apparently rigid bodies, as indicated in Fig. 1, are the two helices to the right and the extra sequence to the *left* of the deep cleft. The extra sequence which in the structure of AK3 complexed to AMP points away from the substrate binding site is part of the active site in the AKe·Ap_5A complex. Assuming the structures of all AKs are similar (7), this means that the extra sequence participates at least indirectly in catalysis. It might serve as a lid that closes off the ATP binding site such that no water can enter this site for hydrolysis. Since short adenylate kinases (AK1) have catalytic activities similar to or even higher than wild-type AKe (see Table III), we have to postulate a different way of shielding the substrate binding site from water.

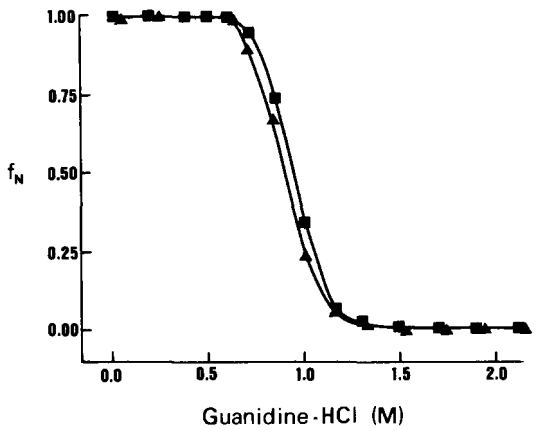

FIG. 8. **Dependence of the fraction of folded protein, f_N, on the concentration of guanidine HCl concentration. ■, wild-type AKe; ▲, $\Delta_{133-157}$ AKe as determined by CD measurements.**

Second, the trypsin digestion experiments also show a different behavior of the mutant enzyme as compared with AKe. The fact that no intermediates accumulated upon the trypsin digestion of wild-type protein but were observed in the case of mutant enzyme seems to indicate that potential sites of tryptic attack in AKe become exposed upon removal of the 133–157 extra sequence. The high sensitivity of $\Delta_{133-157}$ AKe to inactivation by trypsin also raises the question of its resistance to endogenous *E. coli* proteolytic enzymes. The presence in high copy number of the plasmid pEAK1 (>100 at 37 °C) containing the truncated gene probably allows *E. coli* strains temperature-sensitive in the *adk* gene to survive at the nonpermissive temperature with a less active enzyme. It had been shown earlier that an *E. coli* strain carrying a temperature-sensitive *adk* gene on the chromosome and on a plasmid survives at nonpermissive temperatures (3).

Third, although the denaturation temperature determined by differential scanning calorimetry and the denaturation characteristics in guanidinium HCl are very similar for AKe and $\Delta_{133-157}$ AKe, calorimetric experiments indicate a higher $\Delta H^{vH}/\Delta H^{cal}$ ratio for the wild-type AKe. In this context, it should be noted that the van't Hoff enthalpy (ΔH^{vH}) for the wild-type AKe denaturation was determined previously by CD and fluorescence spectroscopy. The ΔH^{vH} values obtained in these earlier studies are very close to the calorimetric enthalpy (ΔH^{cal}) shown here, indicating that protein denaturation is essentially a two-state process. The significantly

TABLE II

Thermodynamic parameters for the unfolding of wild-type and $\Delta_{133-157}$ AKe

T_m is the temperature at which the denaturation of the protein is half-completed. ΔH^{cal} is the calorimetric enthalpy of denaturation calculated from the area under the denaturation curve. ΔH^{vH} is the van't Hoff enthalpy calculated according to the equation. $\Delta H^{vH} = 4RT_m^2\, C_{excess}/\Delta H^{cal}$, where R is the gas constant and C_{excess} is the observed excess specific heat at T_m (26).

Enzyme	T_m	ΔH^{cal}	ΔH^{vH}[a]	$\Delta H^{vH}/\Delta H^{cal}$
	°C	*kcal/mol*		
Wild-type AKe	51.8	95	180	1.9
$\Delta_{133-157}$ AKe	51.5	79	87	1.1

[a] The ΔH^{vH} value shown here for wild-type AK is higher than reported previously (27). The present value represents an average obtained from a larger number of experiments with different batches of protein.

TABLE III

Kinetic parameters of wild-type and deleted AKe and AK1 from rabbit muscle

The reaction medium (0.5 ml final volume) contained either 50 mM Tris-HCl, pH 7.4, 100 mM KCl, 1 mM glucose, 0.4 mM $NADP^+$, 2 mM $MgCl_2$, different concentrations of ADP, and 3 units of each of hexokinase and glucose-6-phosphate dehydrogenase or 50 mM Tris-HCl, pH 7.4, 0.2 mM NADH, 0.5 mM phosphoenolpyruvate, 80 mM KCl 2 mM $MgCl_2$, and 5 units each of lactate dehydrogenase and pyruvate kinase. The reaction was started with enzymes diluted at 2–50 μg/ml in 50 mM Tris-HCl, pH 7.4, supplemented with 1 mg/ml of bovine serum albumin. $K_{m(ADP)}$ 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 0.3 mM (wild-type AKe), 1 mM (rabbit muscle AK1), 1.8 mM ($\Delta_{133-157}$ AKe) 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.

Enzyme	$K_{m(ADP)}$	$V_{max(ADP)}$	$K_{m(ATP)}$	$K_{m(AMP)}$	$V_{max(ATP, AMP)}$
	μM	*μmol/min/ mg of protein*		μM	*μmol/min/ mg of protein*
Wild-type AKe	92	605	51	38	1020
$\Delta_{133-157}$ AKe	420	9.7	1180	1640	74
Rabbit muscle AK1	120	1580	74	86	1800

higher ΔH^{vH} value derived from the calorimetric data (Table II) is likely related to the use of considerably higher concentrations of protein in differential scanning colorimetry experiments than those employed in CD or fluorescence measurements (3, 22). Indeed, the increase in ΔH^{vH} with increasing protein concentration has been noted for other proteins and is believed to reflect a concentration-dependent increase in intermolecular interactions among the protein molecules (23). In this study, the denaturation of wild-type and mutant AKe were compared at similar concentrations. The lower $\Delta H^{vH}/\Delta H^{cal}$ value for the $\Delta_{133-157}$ protein (Table II) suggests that, at least under the conditions of the calorimetric experiments, the degree of intermolecular interaction decreases upon deletion of the (133–157) fragment.

The observation made in this study may be crucial for understanding the role of AK in the metabolism of *E. coli*. Previous genetic and biochemical experiments have suggested that AKe may be directly involved in phospholipid synthesis through formation of a complex with *sn*-glycerol-3-phosphate acyl transferase, a membrane-bound enzyme that catalyzes the first step in phospholipid synthesis (24, 25). The hypothesis that the extra sequence of AKe may be involved in protein-protein and/or in protein-lipid interaction is being investigated in our laboratories.

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REFERENCES

1. Noda, L. (1973) in *The Enzymes* (Boyer, P. D.) 3rd Ed., Vol. 8, pp. 279–305, Academic Press, New York
2. Ray, B. D., Rösch, P., and Nageswara Rao, B. D. (1988) *Biochemistry* **27,** 8669–8676
3. Haase, G. H. W., Brune, M., Reinstein, J., Pai, E. F., Pingoud, A., and Wittinghofer, A. (1989) *J. Mol. Biol.* **207,** 151–162
4. Egner, U., Tomasselli, A. G., and Schulz, G. E. (1987) *J. Mol. Biol.* **195,** 649–658
5. Dreusicke, D., Karplus, P. A., and Schulz, G. E. (1988) *J. Mol. Biol.* **199,** 359–371
6. Müller, C. W., and Schulz, G. E. (1988) *J. Mol. Biol.* **202,** 909–912
7. Schulz, G. E., Müller, C. W., and Diederichs, K. (1990) *J. Mol. Biol.* **213,** 627–630
8. Vetter, I., Reinstein, J., and Rösch, P. (1990) *Biochemistry* **29,** 7459–7467
9. Vetter, I., Konrad, M., and Rösch, P. (1991) *Biochemistry*, in press
10. Brune, M., Schumann, R., and Wittinghofer, A. (1985) *Nucleic Acids Res.* **13,** 7139–7151
11. Taylor, J. W., Oh, J., and Eckstein, F. (1985) *Nucleic Acids Res.* **13,** 8765–8785
12. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, pp. A3 and B20, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
13. Saint Girons, I., Gilles, A.-M., Margarita, D., Michelson, S., Monnot, M., Fermandjian, S., Danchin, A., and Bârzu, O. (1987) *J. Biol. Chem.* **262,** 622–629
14. Chang, J. Y., Brauer, D., and Whittman-Liebold, B. (1978) *FEBS Lett.* **93,** 205–214
15. Yang, P. W., Mantsch, H. H., Arrondo, J. L. R., Saint Girons, I., Gaillou, Y., Cohen, G. N., and Bârzu, O. (1987) *Biochemistry* **26,** 2706–2711
16. Chen, Y. H., Yang, J. T., and Chan, C. H. (1974) *Biochemistry* **13,** 3350–3359
17. Yang, J. T., Chuen-Chang, C. W., and Martinez, H. M. (1986) *Methods Enzymol.* **130,** 208–267
18. Gilles, A.-M., Saint Girons, I., Monnot, M., Fermandjian, S., Michelson, S., and Bârzu, O. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 5798–5802
19. Diederichs, K., and Schulz, G. E. (1990) *Biochemistry* **29,** 8138–8144
20. Koshland, D. E., Jr. (1958) *Proc. Natl. Acad. Sci. U. S. A.* **44,** 98–104
21. Jenks, W. P. (1975) *Adv. Enzymol.* **43,** 219–410
22. Monnot, M., Gilles, A.-M., Saint Girons, I., Michelson, S., Bârzu, O., and Fermandjian, S. (1987) *J. Biol. Chem.* **262,** 2502–2506
23. Hecht, M. H., Sturtevant, J. M., and Sauer, R. T. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 5685–5689
24. Esmon, B. E., Kensil, C. R., Cheng, C.-H. C., and Glaser, M. (1980) *J. Bacteriol.* **141,** 405–408
25. Goelz, S. E., and Cronan, J. E., Jr. (1982) *Biochemistry* **21,** 189–195
26. Sturtevant, J. M. (1987) *Ann. Rev. Phys. Chem.* **38,** 463–488
27. Reinstein, J., Gilles, A.-M., Rose, T., Wittinghofer, A., Saint Girons, I., Bârzu, O., Surewicz, W., and Mantsch, H. H. (1989) *J. Biol. Chem.* **264,** 8107–8112