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Substitution of a serine residue for proline-87 reduces catalytic activity and increases susceptibility to proteolysis of *Escherichia coli* adenylate kinase

(amino acid substitution/thermosensitivity)

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ABSTRACT Amino acid analysis, HPLC separation of trypsin digests, and sequence analysis showed that the thermosensitivity of the adenylate kinase (EC 2.7.4.3) from *Escherichia coli* K-12 strain CR341 T28 results from substitution of a serine residue for proline-87 in the wild-type enzyme. This mutation is accompanied by decreased affinity for nucleotide substrates and decreased catalysis. Circular dichroism spectroscopy showed a significant change of the secondary structure. This mainly corresponds to a reduction in α-helix content (39%) of mutant protein as compared to wild-type adenylate kinase (50%). Altered conformation of thermosensitive adenylate kinase was also manifested by an increase in susceptibility to proteolysis by trypsin. Ap_5A and ATP, known to induce important conformational changes in eukaryotic adenylate kinase(s), protected the mutant enzyme against inactivation by trypsin. This seems to indicate that the "loosening" of the three-dimensional structure of *E. coli* adenylate kinase by proline → serine substitution is largely compensated for when an enzyme·ATP or enzyme·Ap_5A complex is formed.

Adenylate kinase (EC 2.7.4.3) is an essential enzyme that regenerates ADP from ATP and AMP by the reaction MgATP + AMP ⇌ MgADP + ADP. The relatively small size of the enzyme makes it a very interesting model for structural studies. During the last decade, muscle adenylate kinase (myokinase) has been purified from man, pig, rabbit, and calf, crystallized, and investigated extensively by various physicochemical approaches (1–6).

Isolation and genetic characterization of thermosensitive mutants of *Escherichia coli* exhibiting a complex phenotype (defective synthesis of phospholipids, RNA, and ATP at nonpermissive temperatures) allowed localization of mutation to the *adk* gene (7–10). This opened the way for molecular cloning of the *adk* gene, deduction of its primary structure from the nucleotide sequence (11), and overproduction of the protein.

Thermosensitive adenylate kinase of *E. coli* has the same molecular weight as wild-type enzyme but a lower affinity for nucleotides and a V_{max} value that is only 10–15% that of the parental enzyme (12–14). Thermosensitive adenylate kinase is irreversibly inactivated by incubation of crude extracts at 40°C (7, 15–17) due to proteolysis subsequent to thermal denaturation (or transconformation) of mutant enzyme at this temperature (17). It is therefore important to locate the site of mutation to gain a better understanding of the structural factors involved in the maintenance of a stable catalytically active conformation of bacterial adenylate kinase.

In this paper, we show that thermosensitive adenylate kinase differs from wild-type in the substitution of a serine residue for a proline residue at position 87. Some consequences of this mutation for the secondary and tertiary structure of adenylate kinase have been investigated by circular dichroism (CD) spectroscopy as well as by susceptibility to proteolysis by trypsin.

MATERIALS AND METHODS

Chemicals. Adenine nucleotides, substrates, and enzymes were from Boehringer Mannheim. ATP analogs, L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (TPCK-trypsin), and trypsin inhibitor were from Sigma. Urea (fluorimetrically pure) was from Schwartz/Mann. Cibacron blue 3G-A-Sepharose CL-6B (blue-Sepharose) was obtained from Pharmacia (Uppsala, Sweden). Ultrogel AcA 54 came from LKB (France).

Bacterial Strains and Growth Media. *E. coli* strains used in this work were CR341 T28, which is thr^-, leu^-, ile^-, thy^-, $lacY^-$, met^-, *adk*-ts (14), and GT836. GT836 is CR341 T28 transformed by the pIPD37 recombinant plasmid carrying the wild-type adenylate kinase gene. Details concerning the construction of pIPD37 will be published elsewhere. Cells were grown aerobically at 41°C (GT836) or at 28°C (CR341 T28) in LB medium (18) supplemented with 0.01% thymine and ampicillin at 50 μg/ml (GT836) until the late logarithmic phase of growth.

Purification and Assay of Adenylate Kinase. Enzyme from the overproducing strain GT836, in which adenylate kinase is 4% of total cellular protein, was purified as described previously (14). The eluting buffer from the blue-Sepharose step contained 1 mM ATP and 1 mM AMP instead of 0.05 mM P^1,P^5-di(adenosine-5′)-pentaphosphate (Ap_5A). The purification of adenylate kinase from the thermosensitive strain (summarized in Table 1 and Fig. 1) involved, first, chromatography on blue-Sepharose (5 × 30 cm column), the enzyme being eluted with 1 M NaCl in 50 mM Tris·HCl, pH 7.4. After precipitation with solid ammonium sulfate (90% saturation), proteins were redissolved in 50 mM Tris·HCl, pH 7.4, and loaded onto an Ultrogel AcA 54 column (3 × 75 cm) equilibrated with the same buffer. Fractions containing adenylate kinase activity were pooled and loaded directly onto a second blue-Sepharose column (1.5 × 10 cm) equilibrated with 50 mM Tris·HCl, pH 7.4, and eluted with 0.1 mM Ap_5A in the same buffer. Ap_5A was removed from the enzyme by repeated precipitations with ammonium sulfate.

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Table 1. Purification of adenylate kinase from the thermosensitive strain of *E. coli* (CR341 T28)

Step	Protein,* mg	Activity,† μmol/min	Specific activity, μmol/min·mg	Yield, %
Cell extract	11,600	1860	0.16	100
First blue-Sepharose chromatography	1,710	1450	0.85	78
First Ultrogel AcA 54 chromatography	240	1153	4.80	62
Second blue-Sepharose chromatography	17	911	53.5	49
Second Ultrogel AcA 54 chromatography	10.6	619	58.2	33

From 15 liters of culture medium, 94 g of wet cells was collected to start purification.
*Protein concentrations were determined by the procedure of Gornall *et al.* (19) in crude extracts and in the first two steps of purification and by the method of Bradford (20) in the last steps of purification.
†Activity was determined at 27°C in the direction of ATP formation.

The precipitated protein was redissolved in 2 ml of 10 mM ammonium bicarbonate, pH 7.8, and loaded onto a 1.5 × 120 cm column of Ultrogel AcA 54 equilibrated with the same buffer. The purity of the peak fractions containing adenylate kinase was determined by $NaDodSO_4$/polyacrylamide gel electrophoresis. The enzyme solution was kept frozen at −30°C in 10 mM ammonium bicarbonate or was lyophilized. Adenylate kinase activity was determined at 27°C in a final volume of 1 ml by using a spectrophotometric assay system both in the direction of ATP formation and in the direction of ADP formation (14, 17). Absorption changes at 340 nm were followed with a Beckman DU-7 computing spectrophotometer equipped with a temperature-controlled kinetic analysis system and digital printer–plotter. One unit of enzyme activity corresponds to 1 μmol of product formed per min.

Trypsin Digestion, Peptide Separation, Amino Acid Analysis, and Sequence Analysis. Adenylate kinase was carboxymethylated as reported by Waxdal *et al.* (21), then digested at 37°C for 16 hr with 1% (wt/wt) TPCK-trypsin. Peptides were purified by reverse-phase HPLC (DuPont liquid chromatograph 8800) using a column of Lichrosorb RP-8 (Merck) and an ammonium acetate/acetonitrile elution system (22). Manual sequencing of the isolated tryptic peptides or of undigested protein was conducted by the 4-(dimethylamino)azobenzene-4′-isothiocyanate/phenylisothiocyanate double coupling technique (23). Amino acid analyses were performed on a Biotronik LC 5001 amino acid analyzer.

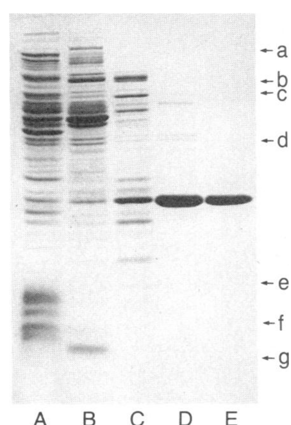

FIG. 1. $NaDodSO_4$/12.5% polyacrylamide gel electrophoresis of fractions obtained during the purification of adenylate kinase from the thermosensitive strain of *E. coli*. The arrows indicate the standard proteins of known molecular weight, from top to bottom: a, phosphorylase *a* (94,000); b, bovine serum albumin (68,000); c, glutamate dehydrogenase (57,500); d, aldolase (40,000); e, β-lactoglobulin (18,000); f, cytochrome *c* (13,000); g, aprotinin (7000). Lane A, bacterial extract (70 μg of protein). Lane B, first blue-Sepharose chromatography (67 μg of protein). Lane C, first Ultrogel AcA 54 chromatography (27 μg of protein). Lane D, second blue-Sepharose chromatography (18 μg of protein). Lane E, second Ultrogel AcA 54 (9 μg of protein).

CD Measurements. The CD spectra were recorded with a Jobin Yvon Mark 3 Dichrograph connected to a Micral 31 microcomputer. Wild-type and mutant adenylate kinase were solubilized in 5 mM ammonium bicarbonate, pH 7.8. Measurements were performed in quartz optical cylindrical cells with 0.01-cm path length, for the spectral range from 260 to 185 nm. Results are expressed in mean residue molar ellipticity, $[\theta]$, in degrees·cm^2·$dmol^{-1}$. For estimation of secondary structure, CD curves in the 190- to 260-nm range were processed by the method of Chen *et al.* (24).

Proteins were measured according to Bradford (20), using pure adenylate kinase ($A_{277}^{1\%}$ = 5.0) as a calibration standard, or by the method of Gornall *et al.* (19), using bovine serum albumin as standard. $NaDodSO_4$/polyacrylamide gel electrophoresis was performed as described by Laemmli (25).

RESULTS

Amino Acid Substitution in Thermosensitive Adenylate Kinase. The amino acid compositions of wild-type and thermosensitive adenylate kinase of *E. coli* are shown in Table 2. There is good agreement between the experimentally determined composition of wild-type adenylate kinase and that deduced from the DNA sequence reported by Brune *et al.* (11). It is clear that the thermosensitive adenylate kinase differs from wild-type enzyme only in the replacement of a proline by a serine residue. To confirm the nature of the mutation and to locate the site of mutation, digests of both

Table 2. Amino acid composition of *E. coli* wild-type and thermosensitive adenylate kinase

Amino acid	Residues per molecule: Wild-type, Calculated	Wild-type, Observed	Thermosensitive, Observed
Cys	1	1.0	1.0
Asx	21	20.9	20.9
Thr	11	11.0*	10.7*
Ser	5	4.9*	5.7*
Glx	26	26.0	26.0
Pro	10	9.7	8.4
Gly	20	18.7	18.7
Ala	19	19.0†	19.0†
Val	19	19.0	19.0
Met	6	4.8	4.8
Ile	14	13.5	13.5
Leu	16	17.1	17.1
Tyr	7	6.5	6.5
Phe	5	5.1	5.1
His	3	4.5	4.2
Lys	18	19.9	20.0
Arg	13	12.4	12.7
Trp	0	ND	ND

Calculated values are from the nucleotide sequence of the *adk* gene (11). Observed values are the means of 10 analyses; residue ratios were calculated for M_r = 23,500. ND, not determined.
*Extrapolated to zero time of hydrolysis.
†Arbitrarily taken as reference value for M_r = 23,500.

forms of adenylate kinase with TPCK-trypsin were separated by reverse-phase HPLC, then subjected to amino acid analysis. The elution profiles of tryptic peptides derived from wild-type or thermosensitive adenylate kinase are very similar (Fig. 2). Peptides were easily identified by their amino acid compositions and some degradation steps. Thus, it appeared that peptide T*12 in thermolabile adenylate kinase was homologous to peptide T12 of wild-type enzyme, in which proline was replaced by a serine residue. The complete sequence of both peptides has allowed localization of the site of amino acid substitution to proline-87.

The adenylate kinase primary structure deduced from the DNA sequence of the cloned *adk* gene (11) was confirmed without ambiguity: (*i*) the N terminus is free and the N-terminal sequence of the first 17 residues of wild-type adenylate kinase corresponded to that predicted by the DNA sequence; (*ii*) all tryptic peptides were recovered after HPLC analysis in good yield. Fig. 3 shows the distribution of tryptic peptides along the amino acid sequence of *E. coli* adenylate kinase as deduced from the DNA sequence.

CD Spectral Analysis. The spectra of wild-type and thermosensitive adenylate kinases are shown in Fig. 4. The patterns are those of proteins with a high α-helix content. However, the signals at ≈222, 208, and 192 nm are weaker in the mutant enzyme, suggesting a lower α-helix content in the thermosensitive adenylate kinase than in the wild-type enzyme. In fact, the secondary structure composition obtained by processing CD curves in the near-UV by the method of Chen *et al.* (24) gave 50% α-helix, 15% β-sheet, and 35% remainder for wild-type adenylate kinase and 39% α-helix, 12% β-sheet, and 49% remainder for thermosensitive adenylate kinase. It should be mentioned that the secondary structure composition of wild-type adenylate kinase of *E. coli* as derived from CD spectra closely resembles that deduced from x-ray values for pig muscle adenylate kinase [55% α-helix, 13% β-sheet, and 32% remainder (1, 26)].

Susceptibility of *E. coli* Adenylate to Proteolysis by Trypsin. Incubation of purified enzymes with trypsin revealed important differences between thermosensitive and wild-type adenylate kinases in their susceptibility to proteolysis (Table 3). We were unable to detect well-defined tryptic fragments on $NaDodSO_4$/polyacrylamide gel electrophoresis, probably because intermediates generated after the first proteolytic cleavage were highly susceptible to further cleavage.

Ap_5A gave by far the greatest protection of both forms of adenylate kinase against trypsin inactivation. ATP and ADP also showed significant protection, whereas AMP was totally ineffective in protecting either adenylate kinase.

DISCUSSION

Site-directed mutagenesis represents a general and powerful method for studying protein function (27). Detection of residues judiciously positioned in the protein structure that play a role in enhanced catalytic activity or stability remains, however, a difficult task despite current knowledge of crys-

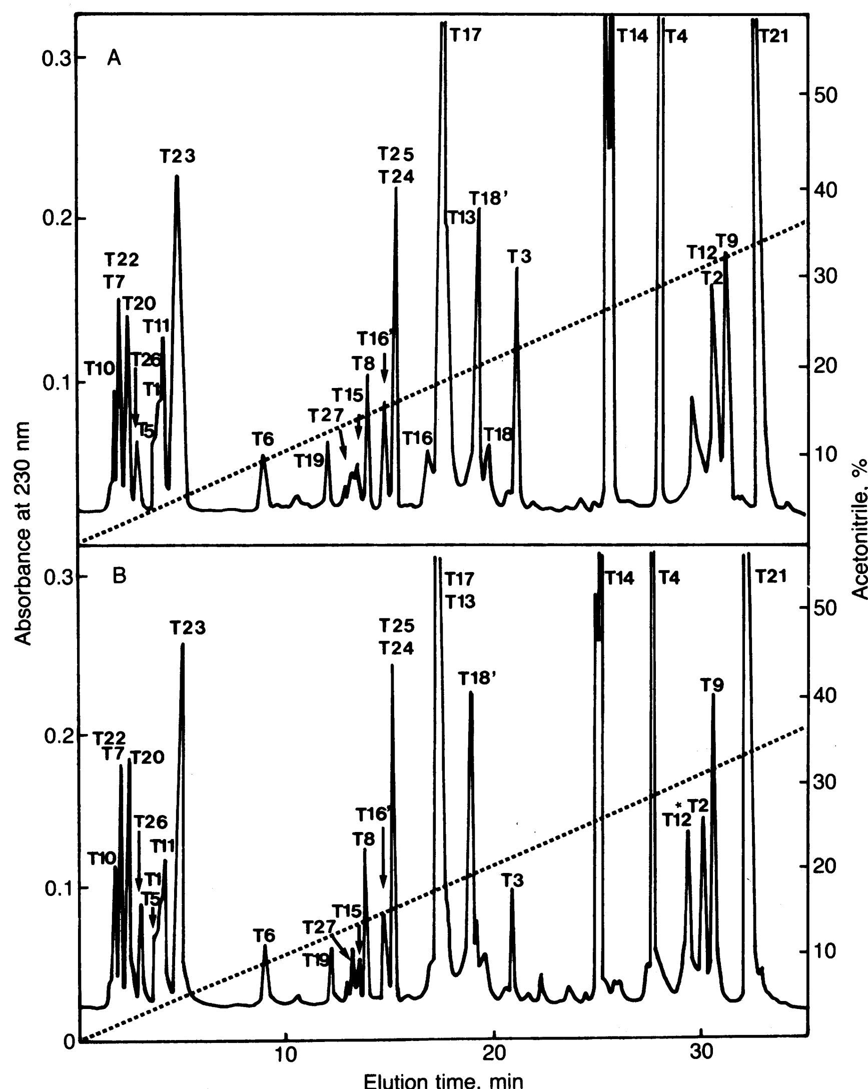

FIG. 2. Separation of tryptic peptides by HPLC on a Lichrosorb RP-8 column. Peptides were eluted with a linear gradient of 0–60% (vol/vol) acetonitrile (---) and detected by their absorbance at 230 nm (—). Peptides are numbered according to their position in the sequence, starting from the N terminus. (*A*) Digest of wild-type adenylate kinase. (*B*) Digest of thermosensitive adenylate kinase. Asterisk indicates the peptide in which an amino acid has been replaced.

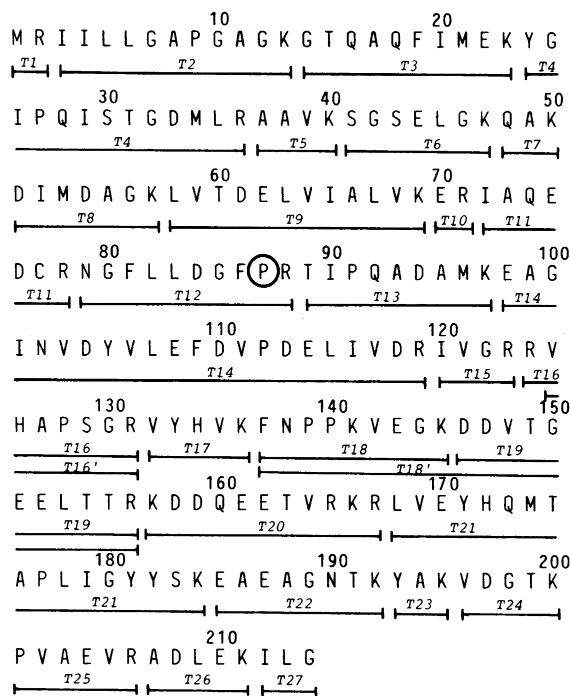

FIG. 3. Distribution of tryptic peptides along the amino acid sequence of *E. coli* adenylate kinase. Amino acid residues are represented by the standard one-letter code. The encircled amino acid (position 87) indicates the site of substitution (proline → serine).

tallographic structures. Therefore, analysis of thermosensitive mutants is extremely illustrative since a single amino acid substitution may be responsible for changes in protein structure and catalytic activity, leading to cell death at nonpermissive temperatures.

Identification of the amino acid substitution in thermosensitive adenylate kinase of *E. coli* CR341 T28 was facilitated by knowledge of the wild-type protein primary structure as deduced from the nucleotide sequence of the *adk* gene (11) as well as development of a reliable purification procedure for mutant enzyme, as described in this paper. The critical step in purification of mutant adenylate kinase is the blue-Sepharose chromatography with specific desorption by Ap_5A at 0.1 mM. Lower concentrations of nucleotide increase the elution volume (2.5–3 column volumes), since mutant adenylate kinase has a lower affinity for Ap_5A than does wild-type enzyme (14). On the other hand, Ap_5A concentrations higher than 0.1 mM increase nonspecific desorption of protein fixed on the blue-Sepharose column.

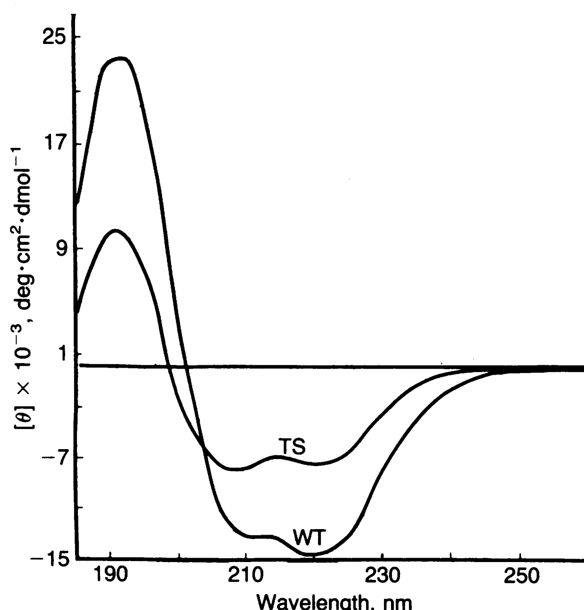

FIG. 4. Mean residue molar ellipticity of wild-type (WT) and thermosensitive (TS) adenylate kinase of *E. coli* in 5 mM ammonium bicarbonate, pH 7.8, at 20°C. Spectra were recorded in 0.01-cm cells at a protein concentration of 0.4 mg/ml.

Table 3. Inactivation of *E. coli* wild-type and thermosensitive adenylate kinase by trypsin and protection by adenine nucleotides

Protective agent	$k \times 10^3$, min^{-1}	
	Wild-type	Thermosensitive
None	23	165
ATP (4 mM)	<1	15
ADP (4 mM)	3	21
AMP (4 mM)	19	170
Ap_5A (0.8 mM)	<1	2

Pure enzyme solution (0.1 ml, 0.5 mg of protein per ml) in 50 mM ammonium bicarbonate, pH 7.8, containing TPCK-trypsin at 1 μg/ml was incubated at 30°C. Nucleotides when present were at the concentrations indicated. At different time intervals (3–60 min), 5-μl aliquots were removed and diluted 1:50 in 50 mM ammonium bicarbonate containing soybean trypsin inhibitor at 25 μg/ml, then residual adenylate kinase activity was determined. Inactivation with trypsin was first order for about 90% of activity loss; rate constants (k) were determined from the slope of semilog plots of enzyme activity versus time. Controls run in the absence of trypsin showed no inactivation of wild-type or thermosensitive adenylate kinase during the 60 min of incubation.

Substitution of a serine residue for proline-87 in wild-type adenylate kinase of *E. coli* decreases both the catalytic constant, k_{cat}, and the affinity for nucleotide substrates (Table 4). Increased susceptibility of mutant enzyme to proteolysis in crude or pure preparations is indicative of alterations of secondary and tertiary structure enhanced by temperature. These structural alterations were confirmed by CD spectra, which showed a lower α-helix content of mutant adenylate kinase as compared to wild-type enzyme.

The following points have to be considered regarding the role of proline-87 in maintaining the catalytically active and thermodynamically stable structure of *E. coli* adenylate kinase:

(*i*) Comparison of *E. coli* adenylate kinase with the pig muscle enzyme and other recently sequenced adenylate kinases (28, 29) showed a rather high degree of homology (about 30%), most evident at the N-terminal half of the molecule. In all these enzymes Pro (conserved as Pro-96 in pig muscle adenylate kinase) is part of an invariant sequence, Gly-Phe-Xaa_h-Xaa_h-Asp-Gly-Xaa_{Ar}-Pro-Arg (Xaa_h represents the hydrophobic residue Ile or Leu, and Xaa_{Ar} represents the aromatic residue Phe or Tyr). In the crystal structure of pig muscle adenylate kinase, the segment involving the first four residues adopts a β-sheet conformation participating in the large active-site cleft (1, 26, 30). The next segment, Gly-Xaa_{Ar}-Pro-Arg followed by another three residues, constitutes a large loop just before an α-helix portion of eight residues. In this position the loop may behave as a hinge capable of governing the cohesion of the protein by permitting ordered portions to come in interactive contacts, leading both to their own stabilization and to stabilization of the whole tertiary structure. Although there is little reason to attribute to Pro-87 a direct role in the catalytic function of *E. coli* adenylate kinase, the conformational and dynamic characteristics of this residue are such that it must confer very particular properties not only to the residues in the loop, but also at a greater distance. Among these residues, at least three (Asp-84, Phe-86, and Arg-88, equivalent to Asp-93,

Table 4. Kinetic parameters of wild-type and thermosensitive adenylate kinase of *E. coli*

		Forward reaction			Reverse reaction				
Strain	Temp, °C	k_{cat}, s^{-1}	K_m^{ADP}, μM	k_{cat}/K_m^{ADP}, $s^{-1} \cdot M^{-1}$	k_{cat}, s^{-1}	K_m^{ATP}, μM	K_m^{AMP}, μM	k_{cat}/K_m^{ATP}, $s^{-1} \cdot M^{-1}$	k_{cat}/K_m^{AMP}, $s^{-1} \cdot M^{-1}$
GT836	27	165	92	1.79×10^6	586	46	38	12.7×10^6	15.4×10^6
	40	319	105	3.04×10^6	1296	46	44	28.1×10^6	29.4×10^6
CR341 T28	27	24	213	0.11×10^6	90	167	155	0.54×10^6	0.58×10^6
	40	39	420	0.09×10^6	131	345	300	0.38×10^6	0.44×10^6

The reaction medium contained, in 1 ml final volume, 50 mM Tris·HCl at pH 7.4, 100 mM KCl, 2 mM $MgCl_2$, nucleotides and coupling enzymes (between 40 μM and 2 mM ADP, 0.4 mM NADP, 1 mM glucose, and 5 units each of hexokinase and glucose-6-phosphate dehydrogenase for the forward reaction; between 25 μM and 1 mM AMP or ATP, 0.1 mM NADH, 1 mM phospho*enol*pyruvate, and 5 units each of pyruvate kinase and lactate dehydrogenase for the reverse reaction). The reaction was started with 5 μl of enzyme solution diluted to the desired concentration (between 2 and 20 $\mu g/ml$). k_{cat} was calculated assuming a molecular weight of 23,500.

Tyr-95, and Arg-97 in pig muscle adenylate kinase) could be implicated in stabilizing interactions in the three-dimensional structure of the protein and in the binding of nucleotides (26). The location of these residues in proximity to the substituted position suggests that the replacement of Pro-87 by the more flexible and polar Ser residue significantly alters their spatial properties. Consequent weakening or disruption of the stabilizing interactions could cause the "loosening" of the mutant protein conformation evidenced by CD experiments.

Changes in kinetic parameters of mutant adenylate kinase are most likely due to destabilization of the protein molecule, which is enhanced by temperature. In fact, temperature increase from 27°C to 40°C had little or no effect on the K_m for nucleotides of wild-type adenylate kinase but caused a 2-fold increase in K_m values for the mutant enzyme (Table 4), in agreement with data reported on partially purified enzyme (13).

(*ii*) Protection of mutant adenylate kinase against proteolytic inactivation by Ap_5A and ATP seems to indicate the ability of these nucleotides to induce a conformational change of the protein molecule that minimizes the possibility of proteolytic attack. In other words, alteration of the three-dimensional structure of adenylate kinase by the Pro → Ser substitution appears to be largely compensated for by the formation of an enzyme–nucleotide complex. Restoration of proper conformation of thermosensitive enzyme by complexing ATP or Ap_5A seems reasonable. In wild-type enzyme the same residues (Asp-84, Phe-86, and Arg-88) participate in both stabilization and binding of nucleotides, as suggested by comparative studies of pig muscle adenylate kinase (26). AMP is unable to induce the same conformational changes, as evidenced by the absence of protection against proteolysis.

Tasks for future research will be to clarify whether or not the role of Pro-87 is unique in maintaining a stable structure of *E. coli* adenylate kinase and to determine if a second mutation could compensate for the three-dimensional structure perturbation induced by the Pro → Ser substitution. Isolation and characterization of thermoresistant pseudorevertants, as well as systematic replacement of Pro-87 by other residues, are expected to provide answers to these questions.

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