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Structural and Functional Consequences of Amino Acid Substitutions in the Second Conserved Loop of *Escherichia coli* Adenylate Kinase*

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Thierry Rose‡, Philippe Glaser§, Witold K. Surewicz¶, Henry H. Mantsch¶, Jochem Reinstein‖, Karine Le Blay‡, Anne-Marie Gilles‡, and Octavian Bârzu‡**

From the ‡Unité de Biochimie des Régulations Cellulaires, §Unité de Régulation de l'Expression Génétique, Institut Pasteur, 75724 Paris Cedex 15, France, the ¶National Research Council of Canada, Ottawa K1A 0R6, Canada, and the ‖Max-Planck Institut für Medizinische Forschung, Abteilung Biophysik, W-6900 Heidelberg, Federal Republic of Germany

All known nucleoside monophosphate kinases contain an invariant sequence Asp-Gly-Phe(Tyr)-Pro-Arg. In order to understand better the structural and functional role of individual amino acid residues belonging to the above sequence, three mutants of *Escherichia coli* adenylate kinase (D84H, G85V, and F86L) were produced by site-directed mutagenesis. Circular dichroism spectra revealed that the secondary structure of all three mutant proteins is very similar to that of the wild-type enzyme. However, each of the substitutions resulted in a decreased thermodynamic stability of the protein, as indicated by differential scanning calorimetry measurements and equilibrium unfolding experiments in guanidine HCl. The destabilizing effect was most pronounced for the G85V mutant, in which case the denaturation temperature was decreased by as much as 11 °C. The catalytic activity of the three mutants represented less than 1% of that of the wild-type enzyme. Furthermore, for the D84H-modified form of adenylate kinase, the impaired binding of nucleotide substrates was accompanied by a markedly decreased affinity for magnesium ion. These observations support the notion that Asp^{84} is directly involved in binding of nucleotide substrates and that this binding is mediated by interaction of the aspartic acid residue with divalent cation. The two remaining residues probed in this study, Gly^{85} and Phe^{86}, belong to a β-turn which appears to play a major role in stabilizing the three-dimensional structure of adenylate kinase.

Adenylate kinase (AK,[1] ATP:AMP phosphotransferase, E.C. 2.7.3.4) plays an essential role in recycling AMP in energetically active cells (1). A relatively small size of the enzyme, together with the availability of an ever-increasing number of mutants obtained via site-directed mutagenesis, render adenylate kinase a suitable model for detailed studies of structure-function relationships in proteins.

Sequence comparison of different isoforms of AK revealed extensive homology, particularly in the N-terminal half of the molecule (2). Thus, the sequence Gly-Xaa-Pro-Gly-Yaa-Gly-Lys-Gly-Thr-Gln, with Xaa standing for Gly, Pro, Ala, or Leu and Yaa standing for Ser or Ala, was found in all forms of AK. This sequence corresponds to residues 15–24 in human, porcine, or rabbit AK1 (3), residues 7–16 in AKe (4), or residues 11–20 in AKy (5). Similar "glycine-rich" loops were found, with some variations, in many other ATP-binding proteins and were shown to be essential for binding of nucleotide substrates (6).

In close spatial proximity to this loop (Fig. 1*A*) there is a second invariant sequence, Gly-Phe-Xaah-Xaah-Asp-Gly-XaaAr-Pro-Arg that corresponds to residues 89–97 in human, porcine, or rabbit AK1, or residues 80–88 in AKe (Xaah stands for the hydrophobic residues Leu, Ile, or Val, and XaaAr stands for the aromatic residue Tyr or Phe). The segment involving the first 4 residues in the latter sequence forms a β-sheet that constitutes an essential part of the large active site cleft. The next segment Gly-XaaAr-Pro-Arg belongs to a β-turn (Fig. 1*B*) that is followed by an 8-residue long α-helix. Previous studies suggested that the 4-residue sequence Gly-XaaAr-Pro-Arg is involved in interactions that stabilize the three-dimensional structure of AKe and is essential for the nucleotide binding. Thus, the substitution of Pro^{87} by Ser decreased the stability of the protein markedly (7, 8), while the substitution of Arg^{88} by Gly resulted in a dramatic loss of enzymatic activity (9).

To further delineate the structural and functional role of residues involved in the "second loop" of AK, we have constructed AKe mutants with Asp^{84} substituted by His, Gly^{85} substituted by Val, and Phe^{86} substituted by Leu, and then analyzed them with respect to their structural properties, their nucleotide binding capability, and their enzymatic activity.

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 typsin and soybean trypsin inhibitor were from Sigma. Blue-Sepharose, polybuffer exchanger 94, and polybuffer 74 were from Pharmacia LKB Biotechnologies Inc. Oligonucleotides were synthesized according to the phosphoamidinate method using a commercial DNA synthesizer (Cyclone™, Biosearch).

Bacterial Strains, Plasmids, and Growth Conditions—Uracil containing single-strand DNA was produced by the ung⁻ dut⁻ *Escherichia coli* strain RZ1032 (10). The *E. coli* strain BMH7118 (11) was used for sequence analysis and AKe overproduction. Plasmid pEAK91

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** To whom correspondence should be addressed: Unité de Biochimie des Régulations Cellulaires, Institut Pasteur, 28, rue du Docteur Roux, 75724 Paris, Cedex 15, France. Tel.: 33-1-45-68-84-05; Fax: 33-1-43-06-98-35.

[1] The abbreviations and trivial name used are: AK, adenylate kinase; AKe and AKy, *E. coli* and yeast adenylate kinase; Ap_5A, P^1,P^5-di(adenosine-5′)pentaphosphate; mAp_5Am, a fluorescent derivative of Ap_5A; blue-Sepharose, Cibacron blue 3G-A-Sepharose CL-6B; HPLC, high performance liquid chromatography; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

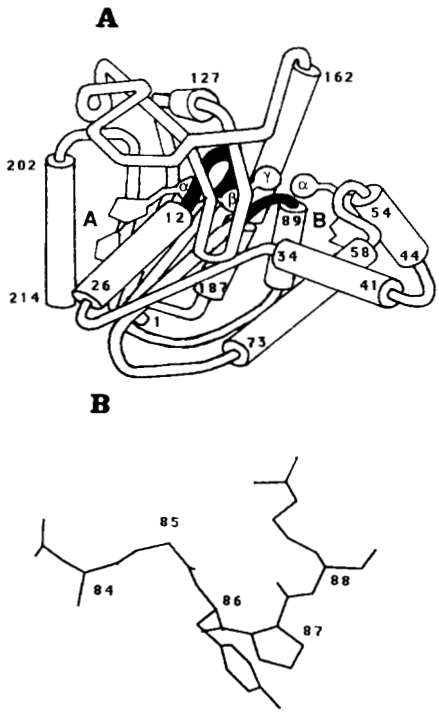

FIG. 1. *A*, schematic drawing of the three-dimensional structure of AKe based on the model of AK1. *Shaded areas* indicate the regions of high homology involved in binding of nucleotide substrates. *B*, computer graphic representation of the polypeptide backbone of residues 84–88 of AKe (corresponding to residues 93–97 in AK1).

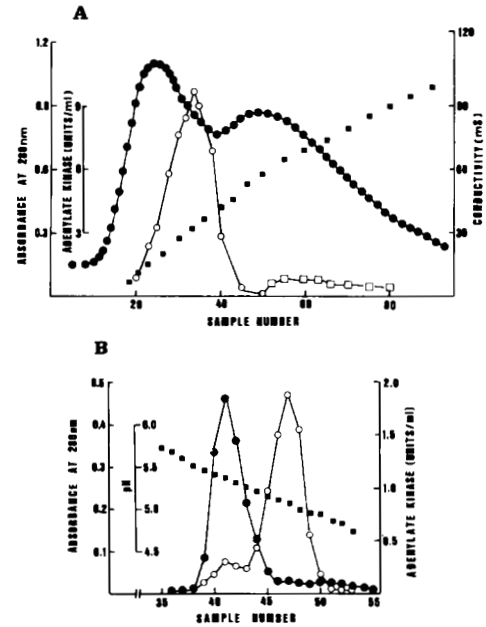

FIG. 2. **Separation of D84H mutant of AKe from the wild-type enzyme by blue-Sepharose chromatography (*A*) or chromatofocusing (*B*).** *A*, the bacterial extract was loaded onto a blue-Sepharose column (25 ml) equilibrated with 50 mM Tris-HCl, pH 7.4, at a ratio of 20 mg of protein/ml of swollen gel. After washing with the same buffer, proteins were eluted with a linear gradient (100 ml in each chamber) of NaCl (up to 1 M, in the same buffer). Fractions of 2 ml were collected at a flow rate of 12 ml/h and analyzed for absorbance at 280 nm (●), conductivity (mS, ■), and adenylate kinase activity in the sense of ATP formation (U/ml, ○, □). *Open circles* correspond to wild-type AKe, *open squares* correspond to D84H mutant. *B*, proteins after Ultrogel AcA-54 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 and analyzed for absorbance at 280 nm (●), activity (units/ml) (○), and pH (■). The first peak of activity coeluting with the bulk of proteins corresponds to the D84H mutant, whereas the second peak of activity corresponds to the wild-type AKe.

overexpressed the *adk* gene under the control of its own promoter (12). Cultures were grown in LB medium (13) supplemented with 100 μg/ml ampicillin. Bacteria were harvested by centrifugation when cultures reached the stationary phase.

Site-directed Mutagenesis and Sequence Analysis—The *Hin*dIII-*Eco*RI restriction fragment from plasmid pEAK91 (13) encompassing the promoter and the 109 first codons from the *adk* gene was subloned in phage M13mp19 (14). Site-directed mutagenesis was performed on the single-strand DNA of the recombinant M13 phage grown in strain RZ1032 as described by Kunkel (15). The aspartic acid (GAC) codon at position 84 was modified to the histidine (CAC) codon using the oligonucleotide 5′AAGCCGTGCAACAGGAA3′. The glycine (GGC) codon at position 85 was modified at the valine (GTC) codon using the oligonucleotide 5′CGCGGGAAGACGTCCAACA3′. The phenylalanine (TTC) codon at position 86 was modified to leucine (CTC) coding using the oligonucleotide 5′TACGCGGGAGGCCGTCCAA3′. The absence of any other mutation in the *Hin*dIII-*Eco*RI fragment was verified by the dideoxynucleotide sequencing method (16). Finally, the original *Hin*dIII-*Eco*RI fragment was replaced by the mutagenized one in plasmid pEAK91 in order to overproduce the modified adenylate kinase(s).

Purification of Adenylate Kinase and Activity Assays—The wild-type enzyme from AKe-overproducting strain of *E. coli* was purified as described previously (17, 18). The modified forms of D84H, G85V and F86L of AKe were purified essentially by the same procedure involving chromatography on blue-Sepharose and Ultrogel AcA-54. The enzyme retained on the 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 1 mM ATP + 1 mM AMP. AKe expressed by the chromosomal wild-type *adk* gene coeluted with G85V and F86L mutants in all types of chromatography including chromatofocussing. The D84H form of AKe was completely separated from the wild-type AKe by salt gradient elution of the enzymes via blue-Sepharose chromatography (Fig. 2*A*) or after chromatofocussing (Fig. 2*B*). Adenylate kinase activity was determined by the coupled spectrophotometric assay (18) at 334 nm and 30 °C in 0.5-ml final volume using an Eppendorf PCP6121 photometer. The reaction medium contained either 50 mM Tris-HCl (pH 7.4), 50 mM KCl, 1 mM glucose, 0.4 mM $NADP^+$, 2 mM $MgCl_2$, different concentrations of ADP, and 2 units each of hexokinase and glucose-6-phosphate dehydrogenase, or 50 mM Tris-HCl (pH 7.4), 100 mM KCl, 2 mM $MgCl_2$, 1 mM phosphoenolpyruvate, 0.1 mM NADH, different concentrations of AMP and ATP, and 2.5 units each of pyruvate kinase and lactate dehydrogenase. The reaction was started with 3–5 μl of adenylate kinase diluted to 2–100 μg/ml with 50 mM Tris-HCl (pH 7.4). 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 AKe—Wild-type and modified forms of AKe were digested for 15 h at 37 °C with TPCK-trypsin (1% w/w). Peptides were purified by reversed-phase HPLC on a Perkin-Elmer instrument (series 410) equipped with an LC135 diode array detector, using a Nucleosil C-18 column (5 μm, 4.6 × 250 mm) and an ammonium acetate pH 6/acetonitrile elution system at a flow rate of 1 ml/min. Fractions were identified by absorbance recording at 230 and 280 nm. Amino acid analysis of peptides was performed on a Beckman System 6300 high performance analyzer after 6 N HCl hydrolysis for 22 h at 110 °C.

Spectroscopic Studies—Circular dichroism (CD) spectra were acquired at room temperature on a Jasco-600 spectropolarimeter. Samples at protein concentration of approximately 0.4 mg/ml were placed in a 0.2-mm cylindrical cuvette and, typically, eight spectra were averaged to improve the signal-to-noise ratio. Secondary structure estimations were obtained by processing CD curves between 180 and 250 nm using the software provided by Jasco. Protein concentrations used to normalize the spectra were determined by amino acid analysis. The equilibrium unfolding of AKe as a function of guanidine HCl concentration was studied at room temperature by monitoring the loss of ellipticity at 222 nm. Samples for these measurements were prepared by mixing 0.04 ml of the stock protein solution (2 mg/ml) with 0.16 ml of guanidine HCl solution at a desired concentration. All samples were equilibrated for 12 h at 22 °C before measurements

were made. The fraction of protein folded, f_N, was calculated as $f_N = (\Theta - \Theta_u)/(\Theta_N - \Theta_u)$, where Θ is the observed ellipticity at 222 nm at some point in the transition and Θ_N and Θ_u are the values for the native and unfolded forms, respectively. Values for Θ_N and Θ_u in the transition zone were determined by linear extrapolation.

Binding of ATP and ADP to AKe was investigated by determining the fluorescence enhancement of mAp_5Am upon binding to enzyme in the absence or presence of nucleoside di- and triphosphates. Fluorescence measurements (excitation wavelength 360 nm, emission wavelength 440 nm) were performed with an SLM "Smart" 8000 photon-counting spectrofluorimeter. The dissociation constant of the AKe/mAp_5Am complex was determined from the fluorescence titration of each particular protein with the nucleotide analog, and was used to calculate the dissociation constant for ATP or ADP (19).

Differential Scanning Calorimetry—The thermal stability of wild-type AKe and of the mutant proteins was studied by differential scanning calorimetry using an ultrasensitive Microcal MC-2D instrument at a scanning rate of 50 °C/h. Protein concentrations were in the range of 1.5–2.5 mg/ml in 0.05 M HEPES, pH 7.2. Data from DSC experiments were analyzed employing the software provided by Microcal Inc., Northampton, Md.

RESULTS

Purification of D84H-, G85V-, and F86L-modified Forms of AKe—The soluble extract from *E. coli* expressing mutated *adk* genes on plasmid pEAK91 exhibited under standard assay conditions (30 °C, pH 7.4, and 1 mM ADP as substrate) an activity close to that of the same *E. coli* strain without plasmid (about 1 unit/mg of protein). After purification, the G85V and F86L forms of AKe (30% of the total *E. coli* proteins) exhibited a specific activity of 5.1 and 8.8 units/mg of protein, respectively. Wild-type AKe expressed by the chromosomal gene (about 0.1% of *E. coli* proteins) coeluted with these modified forms of bacterial enzyme. We assume that the specific activity of G85V and F86L mutants is at most 0.5% of the specific activity of wild-type AKe and that their "contamination" by wild-type AKe is in the same range. Since attempts to separate these modified forms from the wild-type AKe failed we did not examine their kinetic properties. Purified G85V and F86L forms of AKe were suitable for structural and nucleotide binding studies. The D84H mutant on the other hand had a higher pI than the wild-type enzyme. The separation of this mutant could be achieved by either salt gradient elution from blue-Sepharose step (Fig. 2*A*) or by chromatofocussing (Fig. 2*B*). The results of amino acid analysis of the modified forms of AKe were in good agreement with those expected for AKe with substitution of Asp^{84} by His, of Gly^{85} by Val, and of Phe^{86} by Leu, respectively (Table I).

Secondary Structure of Mutant Proteins—The effect of single amino acid substitutions on the secondary structure of AKe was studied by CD spectroscopy. As reported previously (20), the far-UV CD spectrum of wild-type AKe is characterized by negative minima at approximately 222 and 208 nm, which is indicative of a high content of α-helical structure. The CD spectra of the three investigated mutants are very similar to that of the wild-type enzyme (Fig. 3). Quantitatively, the largest difference in the spectral intensity was found between wild-type protein and the F86L mutant. However, even for the latter protein, the estimated content of a α-helix is only 3% below that calculated for the wild-type. Thus, it may be concluded that none of the substitutions made affected the secondary structure of the enzyme significantly.

Stability of Mutant Proteins—Excess heat capacity curves for the wild-type AKe and the D84H, G85V, and F86L mutants are shown in Fig. 4. These curves were obtained by subtracting the base lines from raw calorimetric data using a cubic splines interpolation procedure. Inspection of Fig. 4 reveals that the mutants studied all have a decreased thermal stability. The destabilization effect is most pronounced for the G85V mutant. The unfolding of the latter protein is essentially completed at the temperature at which the wild-type enzyme is still in the fully native state. Each of calorimetric curves could be approximated by a single transition. The mean denaturation parameters derived from two to three experiments with each protein are listed in Table II. While

TABLE I

Amino acid composition of E. coli-modified adenylate kinases and of the wild-type protein

Amino acid	D84H mutant	G85V mutant	F86L mutant	Wild-type adenylate kinase	
				Observed	Calculated[a]
Cys	ND[b]	ND[b]	ND[b]	ND[b]	1
Asx	19.3	20.1	19.8	19.9	21
Thr	9.7[c]	9.5[c]	9.4[c]	9.6[c]	11
Ser	4.4[c]	4.4[c]	4.1[c]	4.1[c]	5
Glx	25.5	25.2	24.2	25.0	26
Pro	9.8	9.8	9.6	10.3	10
Gly	19.7	18.7	19.4	19.6	20
Ala	19.0[d]	19.0[d]	19.0[d]	19.0[d]	19
Val	18.4	19.1	18.1	18.2	19
Met	ND[b]	ND[b]	ND[b]	ND[b]	6
Ile	12.4	12.3	12.3	12.3	14
Leu	16.5	16.3	17.1	16.1	16
Tyr	5.9[c]	5.9[c]	6.0[c]	5.9[c]	7
Phe	5.0	4.9	3.8	4.8	5
His	4.2	3.3	3.1	3.1	3
Lys	17.4	17.3	17.0	17.1	18
Arg	12.7	12.6	12.8	12.6	13

[a] From the nucleotide sequence of the *adk* gene (4).
[b] ND, not determined.
[c] Uncorrected values determined after 20 h of hydrolysis.
[d] Arbitrarily taken as reference value for M_r = 23,500.

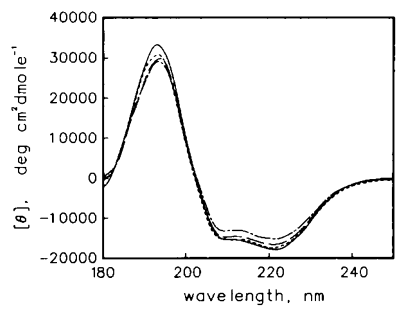

FIG. 3. **Circular dichroism spectra in the far UV region for wild-type AKe (*solid line*), D84V (···), G85V (– –), and F86L (– – –) mutants.**

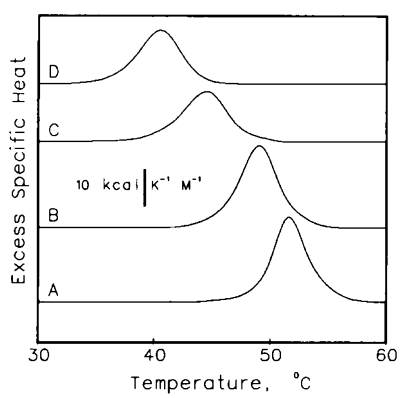

FIG. 4. **Differential scanning calorimetric traces for wild-type AKe (*A*), F86L (*B*), D84H (*C*), and G85V (*D*) mutants.** The *curves* presented were obtained by smoothing raw calorimetric data and substracting from them the base lines using a cubic interpolation procedure.

TABLE II

Calorimetric parameters for denaturation of the wild-type and mutant adenylate kinase

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. $\Delta\Delta G°$ is the standard free energy of denaturation corrected to T_m of the wild-type protein, calculated using the Gibbs-Helmholtz equation (25). Since repetitive experiments gave no consistent indication of heat capacity differences between the native and denatured proteins, the enthalpy of denaturation used in calculating $\Delta G°$ was assumed to be independent of temperature.

Enzyme	T_m	ΔH_{cal}	$\Delta\Delta G°$
	°C	*kcal/M*	
AKe wild-type	51.8	95	0
$Asp^{84}His$	44.6	63	1.4
$Gly^{85}Val$	40.7	70	2.4
$Phe^{86}Leu$	49.2	97	0.8

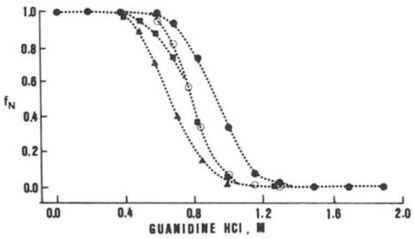

FIG. 5. **Dependence of the apparent fraction of folded proteins f_N, on the guanidine-HCl concentration for the wild-type AKe (●), D84H (■), G85V (▲), and F86L (○) mutants.**

T_m values were reproducible within less than 0.3 °C, the uncertainty in determining the calorimetric enthalpy of denaturation is estimated to be as large as 15–20%. The relatively large errors in ΔH_{cal} reflect the difficulty in precise determination of calorimetric base lines. Consistent with previous observations for wild-type AKe and its R88G mutant (9), the ratio of van't Hoff enthalpy to calorimetric enthalpy was found to be significantly above 1 for all proteins studied. This suggests the existence of intermolecular interactions, most likely due to protein self-association (21, 22). An additional parameter derived from calorimetric data is the standard free energy of denaturation calculated at 51.8 °C, the T_m for the wild-type. The latter value (last column in Table II) may be interpreted as a standard free energy of destabilization of each mutant protein relative to the wild-type enzyme (23–25).

The reduced stability of the mutant proteins was corroborated by equilibrium unfolding experiments in guanidine HCl. For all proteins, the dependence of the fraction folded, f_N, on the denaturant concentration (Fig. 5) indicates a two-state unfolding process. The relative destabilizing potency of different amino acid substitutions as indicated by the chemical denaturation experiments is in general agreement with that deduced from calorimetric measurements. The least satisfactory correlation is found for the F86L AKe. While by calorimetric criteria this protein is more stable than all other mutants studied (except the wild-type), its sensitivity to chemical denaturation is very close to that of D84H AKe. The reason for this slight discrepancy is at present unknown.

Proteolysis of AKe Mutants by Trypsin—The susceptibility of native proteins to inactivation by proteases is a sensitive probe of conformational changes induced by ligands by physical or chemical agents, or by amino acid substitutions via site-directed mutagenesis. The first order rate constant of inactivation of AKe by TPCK-trypsin at pH 7.4 and 30 °C (0.5×10^{-3}/s) was in good agreement with the decrease in absorbtion of the AKe band scanned after sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie Blue staining. Under identical experimental conditions the D84H, G85V, and F86L mutants showed close sensitivity against proteolysis by trypsin (calculated k_1 values were between 0.5 and 0.7×10^{-3}/s). It is interesting to note, however, that at 40 °C, a temperature which corresponds to T_m of the G85V form of AKe, the mutant enzyme was proteolyzed four times faster ($k_1 = 1.2 \times 10^{-2}$/s) than the wild-type protein ($k_1 = 2.9 \times 10^{-3}$/s) (Fig. 6).

Kinetic and Nucleotide-binding Properties of the Modified Forms of AKe—Preliminary assays under standard conditions (1 mM ADP or 1 mM ATP + 0.3 mM AMP) of pure D84H form of AKe indicated that its activity is 1% of that of wild-type protein. At 20 mM Mg^{2+} and 5 mM nucleotide substrate the reaction rate represented 1.5% (in the sense of ATP formation) and 4.4% (in the sense of ADP formation) of that of the wild-type protein. Table III shows the kinetic parameters for the wild-type and D84H mutant AKe. The apparent K_m for nucleotides was increased by a factor of 5 to 11 in the D84H form of AKe. The most striking differences between the wild-type and D84H-modified form of AKe was a higher requirement of Mg^{2+} of the latter to reach maximal activity (Fig. 7).

The two other modified forms of AKe (G85V and F86L) exhibited activities which represented at most 0.5% of that of

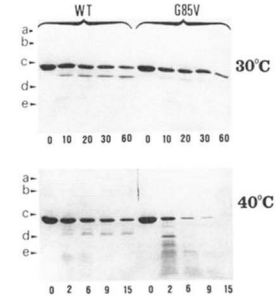

FIG. 6. **Proteolysis of wild-type AKe and of the G85V mutant by trypsin.** Enzymes at 1 mg/ml in 50 mM Tris-HCl (pH 7.4) and 50 mM KCl were incubated at 30 and 40 °C with TPCK-trypsin (2 μg/ml). At different time intervals (indicated in minutes at the bottom of each lane), 20-μl aliquots were withdrawn, boiled with electrophoresis buffer, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5%) and Coomassie Blue staining. The molecular weight standards, from top to bottom are: *a*, bovine serum albumin (67,000); *b*, ovalbumin (43,000); *c*, carbonic anhydrase (30,000); *d*, soybean trypsin inhibitor (20,100); *e*, lysozyme (14,400).

TABLE III

Kinetic parameters of wild-type and D84H mutant adenylate kinase of E. coli

The reaction conditions are described under "Experimental Procedures." The concentration of Mg^{2+} was 2 mM (wild-type AKe) and 20 mM (D84H mutant), respectively. K_m^{ADP} and V_m^{ADP} were determined from plots of $1/v$ *vs.* $1/ADP^2$, 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 were determined at a single final concentration of cosubstrate (0.3 mM AMP for wild-type AKe, 1 mM AMP for D84H mutant, and 1 mM ATP for both forms of enzyme). The $V_m^{ATP,AMP}$ was obtained by extrapolating the reaction rates for infinite concentration of ATP and AMP and assuming that the concentration of one nucleotide substrate does not affect the apparent K_m of the enzyme for the second nucleotide substrate.

Enzyme	K_m^{ADP}	V_m^{ADP}	K_m^{ATP}	K_m^{AMP}	$V_m^{ATP,AMP}$
	μM	*μmol/min/mg of protein*	μM		*μmol/min/mg of protein*
Wild-type	92	605	51	38	1020
D84H mutant	870	8.8	270	430	45

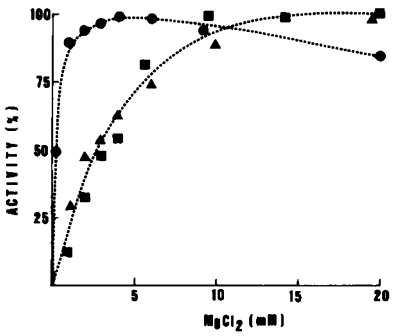

FIG. 7. **Dependence of adenylate kinase activity on the concentration of Mg^{2+}.** The experiments were performed on wild-type enzyme (●) in the sense of ATP formation and on the D84H mutant both in the sense of ATP (▲) or ADP formation (■). The nucleotide concentration was held constant (1 mM ADP, 1 mM ATP and 0.3 mM AMP, respectively).

TABLE IV

Dissociation constants of mAp_5Am, ATP, and ADP to wild-type and modified form of adenylate kinase of E. coli

50 mM Tris-HCl (pH 7.5) containing 5 mM $MgCl_2$ or 2.5 mM EDTA, 0.3 μM mAp_5Am, and different amounts of ATP or ADP at a final volume of 1 ml, were titrated with increasing concentrations of protein, using λ_{ex} = 360 nm and λ_{em} = 440 nm. Calculation of K_d values from fluorescent experiments were made according to Ref. 19.

Enzyme	Mg-mAp_5Am	EDTA-mAp_5Am	Mg-ATP	EDTA-ADP
		μM		
Wild-type	0.3	0.16	85	4.0
G85V mutant	6.0	1.39	480	5.1
F86L mutant	5.1	1.03	78	3.7

wild-type protein. Traces of the wild-type protein expressed by the chromosomal *adk* gene present in preparations of modified AKe did not allow accurate kinetic analysis of these enzymes. Use of a fluorescent analogue of Ap_5A enabled us, however, to determine the affinity of these enzymes for the nucleotide substrates, ATP and ADP, respectively (Table IV). Both G85V and F86L mutants had a significantly lower affinity for mAp_5Am. However binding of ATP and of ADP was not affected in the case of the F86L mutant, whereas in the case of the G85V mutant the affinity for ATP was decreased by a factor of 5.6 as compared to the wild-type protein.

DISCUSSION

A considerable effort has been made over the past few years to elucidate structure-function relationships in adenylate kinase (26–33). Particular emphasis has been placed on identification and characterization of the structural elements that are essential for nucleotide binding and enzymatic activity of the protein. In this study, we have focussed on the second loop in the *E. coli* adenylate kinase that comprises residues ^{84}Asp-Gly-Phe-Pro-Arg^{88}. Crystallographic data have shown that the last 4 of these 5 residues belong to a type VIb β-turn inserted between a β-strand and an α-helical segment (34). As the residues in question are highly conserved in all known isoforms of adenylate kinase (and likely also in other nucleoside monophosphate kinases), this sequence is anticipated to play a crucial role in the catalytic transfer of the phosphoryl group from the nucleotide donor to the nucleotide acceptor.

The results of this study, together with earlier data obtained for Pro^{87} (7, 8) and Arg^{88} (9) mutants of AKe, indicate that each of the residues belonging to the ^{84}Asp-Gly-Phe-Pro-Arg^{88} segment is critically important for maintaining the structural integrity and/or catalytic function of the enzyme. The effects produced by amino acid substitutions within the Asp^{84}–Arg^{88} region may be categorized into two classes: (i) a decrease in the catalytic activity (20–100-fold inactivation was found for all mutants except the Pro^{87}Ser enzyme, for which the inactivation was less severe), and (ii) a decrease in the thermodynamic stability of the protein. The latter effect is particularly pronounced for the enzymes containing substitutions at positions Gly^{85} or Pro^{87}.

From the point of view of protein function, the most interesting effect was observed upon replacement of Asp^{84} by histidine. In addition to significantly lowering the V_m value and increasing K_m for all nucleotide substrates, this substitution resulted also in a markedly reduced affinity of the enzyme for Mg^{2+}. This decrease in affinity of the Asp^{84}His mutant protein for divalent cations strongly indicates that Asp^{84} participates directly in the coordination of the metal ion. The above picture is consistent with the x-ray data for AK cocrystallized with Ap_5A (35, 36) as well as with the recent NMR results for chicken muscle adenylate kinase (37). In the latter case, the replacement of Asp^{93} (the residue equivalent to Asp^{84} in AKe) with alanine was found to impair the binding of Mg^{2+} ions.

The two other residues probed in this study, Gly^{85} and Phe^{86}, are part of the β-turn. Substitution of each individual amino acid within the turn sequence ^{85}Gly-Phe-Pro-Arg^{88} results in thermodynamic destabilization of AKe. The degree of this destabilization varies considerably, being most pronounced for mutants Gly^{85}Val and Pro^{87}Ser. A large disordering effect of the Pro^{87} → Ser substitution has been rationalized previously (7). In the case of Gly^{85}Val mutant, replacement of the hydrogen atom at C_α of glycine by a more bulky side chain of valine imposes serious steric constraints which likely distort the β-turn and may lead to an overall destabilization of the protein structure. Although certain details require further clarification, the present data allow us to postulate that the decrease in catalytic activity of AKe upon amino acid substitutions within the turn 85 → 88 region arises as a consequence of an overall destabilization of the native protein structure.

A point worth brief discussion is the comparison of structural perturbations produced by mutation G^{85}V with those reported previously for the Pro^{87}Ser protein. While both substitutions have comparable effects on the thermodynamic stability of AKe (as indicated by a similar drop in the denaturation temperature), the susceptibility of the two mutant proteins to proteolytic digestion by trypsin is quite different. Thus, at 30 °C, *i.e.* a temperature below the denaturation temperature of both proteins, the G^{85}V form showed resistance to proteolytic digestion similar to that of the wild-type enzyme, whereas the Pro^{87}Ser mutant was proteolyzed at a greatly increased rate. This suggests that the perturbation introduced by the Pro → Ser substitution is more drastic and is likely to result in a considerably altered tertiary and/or secondary structure of the protein. Indeed, far UV circular dichroism spectra point to a virtually unchanged secondary structure of the G^{85}V protein, while the Pro^{87}Ser mutants shows a significantly reduced content of α-helix (7).

Phenylalanine at position 86 (or equivalent) alternates with tyrosine in all nucleoside monophosphate kinases. The functional equivalence of these 2 residues has been demonstrated directly by the Tyr → Phe mutation in human muscle AK1. The above two forms of adenylate kinase were found to have similar catalytic and thermodynamic properties (38). In this context, it is worthwhile noting that replacement of Phe^{86} in *E. coli* adenylate kinase by another aromatic side chain, Trp, led to an enzyme with normal thermal stability and maximal catalytic activity, but with an altered affinity for ATP (a 3-

fold increase in K_m) and for AMP (a 44-fold increase in K_m) (39). On the other hand, Phe[86]Leu substitution resulted in an almost completely inactive protein, as demonstrated recently by Liang *et al.* (39) and corroborated in this study. Regarding the latter enzyme, we find it somewhat surprising that the Phe[86]Leu mutation had only a modest effect on the overall thermodynamic stability of adenylate kinase. Statistical studies with model peptides postulate the "locking effect" of the phenyl group of Tyr or Phe side chains on the conformation of proline (40, 41). One could thus expect the Phe → Leu substitution to perturb the *cis* conformation of the nearby Pro[87] in AKe, rendering a considerably less stable enzyme. This is, however, not the case.

In summary, the results of this study, together with the data obtained recently by other groups (37, 40), indicate that the highly conserved DGYPR sequence situated in close proximity to the enzyme-bound ATP or AMP contributes to the formation and stabilization of the AK/AMP/ATP ternary complex via Mg^{2+} ion. While Asp[84] participates directly in the complexation of magnesium ions, the next 4 residues are involved in this β-turn. This turn appears to play a crucial stabilizing role in the enzyme, contributing thus indirectly to the catalytic step.

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