Structurally and Catalytically Important Residues in the Phosphate Binding Loop of Adenylate Kinase of *Escherichia coli*

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Received August 16, 1989; Revised Manuscript Received April 2, 1990

ABSTRACT: Amino acids in the phosphate binding loop of adenylate kinase of *Escherichia coli* were mutated by site-directed mutagenesis. The mutant proteins with a Pro-9 → Gly (P9G) and with a Lys-13 → Gln (K13Q) exchange were overexpressed and purified. They were characterized by steady-state kinetics, fluorescence binding, and structural studies, together with the phosphate binding loop mutants P9L and G10V prepared earlier [Reinstein, J., Brune, M., & Wittinghofer, A. (1988) *Biochemistry 27*, 4712–4720]. The results obtained show that all these mutations change the structure of the protein as evidenced by NMR spectroscopy and temperature-stability studies. All the mutant proteins have increased dissociation constants for substrates and inhibitors, but their catalytic activity, except for K13Q, is not reduced. The results obtained with K13Q suggest that this lysine residue, which is conserved in all guanine and many adenine nucleotide proteins, might have an important role in catalysis.

Many proteins that bind and enzymatically process guanine, adenine, or uridine (and probably other) nucleotides contain a consensus sequence in their primary structure which can be written as GXXXXGK(S/T). From the crystal structure analysis of the guanine nucleotide binding proteins EF-Tu and p21 and the adenine nucleotide binding protein adenylate kinase, it is clear that this structural motif constitutes a loop that wraps around a phosphate group (Egner et al., 1987; Dreusicke & Schulz, 1986; Jurnak, 1985; LaCour et al., 1985; De Vos et al., 1988; Pai et al., 1989). This phosphate group is the β-phosphate of GDP and GppNp in p21 complexes (de Vos et al., 1988; Pai et al., 1989) and phosphate 2 of the adenylate kinase–AP5A[1] complex (Egner et al., 1987; Müller & Schulz, 1988). Figure 1 shows the 3-D structure of this phosphate binding loop of *Escherichia coli* adenylate kinase together with AP5A (the coordinates are a generous gift of G. E. Schulz).

On more detailed analysis one finds some differences between the phosphate binding motifs of G-binding proteins on the one hand and adenylate kinases or other ATP-converting enzymes like F_1-ATPase (Rao et al., 1988) and UMP–ATP phosphotransferases (Wiesmüller et al., 1990) on the other hand.

The guanine nucleotide binding proteins of the ras gene family contain the following consensus sequence

Gly-Xaa-Gly*-Gly-Val- -Gly-Lys- -Ser

whereas the adenylate kinases contain

Gly-Xaa-Pro*-Gly-Ala/Ser-Gly-Lys-Gly-Thr

in the corresponding region. Interestingly it has been found for p21 (the product of the ras gene) that the replacement of glycine in position 12 (marked by an asterisk) with any other amino acid except proline renders the protein oncogenic (Seeburg et al., 1984). In adenylate kinases this residue is a conserved proline. It was thus of great interest to mutate this conserved proline residue to glycine and monitor the concomitant effect on the structure and function of adenylate kinase in order to compare the effect to the corresponding mutation in p21.

The other highly conserved residue in both families of proteins is lysine, which in the crystal structure has been shown to point with its side chain toward the phosphate groups (Egner et al., 1987; Müller & Schulz, 1988; Pai et al., 1989) as shown in Figure 1 for AKec. From the structure analysis alone one cannot decide what the exact function of this residue is: Does it merely serve to stabilize this tight loop, which connects a β-sheet with an α-helix as inferred from crystallographic analysis? Is it involved in stable binding of one or more of the phosphate groups in the "giant anion hole" (Dreusicke & Schulz, 1986) formed by the main-chain nitrogens and this lysine residue? Is it involved in the catalytic step of phosphoryl transfer? The corresponding lysine residue of AK1 (chicken) has been found to be chemically modified by substrate analogues like adenosine diphosphopyridoxal, and the modified enzyme was totally inactive (Tagaya et al., 1987). Substrates like ATP or ADP (and to a small extent AMP) protect against this inactivation. Corresponding lysine residues in this consensus region have also been mutated in F_1-ATPase (Rao et al., 1988) and in Rho protein (Dombroski et al., 1988), resulting in loss of activity of these proteins. However, none of the questions about its specific role have been addressed. In the present work we mutated Lys-13 to Gln and investigated the properties of the resulting mutant protein by kinetic and structural (NMR) techniques. We extend these measurements to mutant proteins containing the Pro-9 → Leu (P9L) and Gly-10 → Val (G10V) mutations in the phosphate binding loop already described and compare them to results obtained earlier (Reinstein et al., 1988).

Materials and Methods

Strains and Plasmids. SMH50 (Taylor et al., 1985) was obtained from F. Eckstein. The plasmid pEAK90, which

[1] Abbreviations: AK, adenylate kinase (EC 2.7.4.3); AKec, adenylate kinase of *Escherichia coli*; AK1, mammalian cytosolic enzyme; AMP, ADP, and ATP, adenosine 5′-mono, 5′-di-, and 5′-triphosphate; AP*n*A, P^1,P^n-di(adenosine-5′) *n*-phosphate; mAP5Am, α,ω-di[(3′ or 2′)-*O*-(*N*-methylanthraniloyl)adenosine-5′] pentaphosphate; mAP5A, α-[(3′ or 2′)-*O*-(*N*-methylanthraniloyl)adenosine-5′] ω-(adenosine-5′) pentaphosphate; mdATP, 3′-*O*-(*N*-methylanthraniloyl)-2′-deoxyadenosine 5′-triphosphate; EDTA, ethylenediaminetetraacetate; CD, circular dichroism; NMR, nuclear magnetic resonance; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate. Mutant adenylate kinases from *E. coli*: P9G, Pro-9 → Gly exchange; P9L, Pro-9 → Leu exchange; G10V, Gly-10 → Val exchange; K13Q, Lys-13 → Gln exchange.

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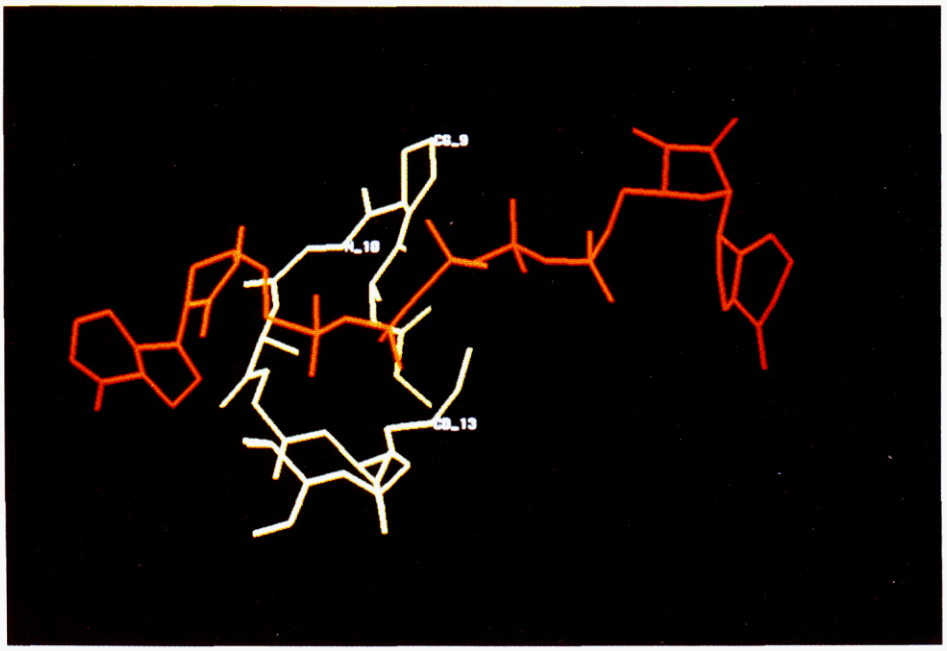

FIGURE 1: Structure of AKec–AP5A complex showing part of the polypeptide chain of adenylate kinase of *E. coli* with AP5A taken from the 3-D structure of the AKec–AP5A complex (Müller & Schulz, 1988). The bisubstrate inhibitor AP5A is shown in red, and amino acids 7–15 (the "phosphate binding loop") are shown in green (sequence: $_{7}$GA*P*GAG*K*GT$_{15}$). The amino acids Pro-9, Gly-10, and Lys-13, which have been mutated in this study, are marked by white labels. The part of AP5A which is on the left side of the picture is referred to as the ATP site and that on the right side as the AMP site (Vetter et al., 1989). Coordinates of the X-ray structure were a generous gift of G. E. Schulz, Freiburg, FRG.

contains the adk gene, is a derivative of pEMBL9 (Dente et al., 1983) and was described earlier (Reinstein et al., 1988).

Cloning Methods. Restriction enzymes, deoxynucleotides, and T4 DNA ligase were obtained from Boehringer Mannheim. DNA polymerase I large fragment (Klenow fragment) was from NEN. Enzyme buffers were used according to Maniatis et al. (1982). For transformation with DNA, cells were treated with $CaCl_2$ according to Mandel and Higa (1970).

Synthesis and Purification of Oligonucleotides. Oligonucleotides were synthesized according to the phosphoamidite method with a commercial DNA synthesizer (Cyclone Biosearch). Products were purified in a two-step procedure with HPLC (Beckman) using a C18-ODS column and the following parameters; flow rate, 2 mL/min; elution gradient, 20–40% acetonitrile for 20 min in 100 mM triethylammonium acetate (TEA) for the dimethoxytrityl-protected oligonucleotides and in 5–20% acetonitrile for 30 min for the detritylated oligonucleotides.

Site-Directed Mutagenesis. The mutagenesis procedure was carried out with pEAK90 containing the adk gene according to the thio method of Taylor et al. (1985) essentially as described in Reinstein et al. (1988). The oligonucleotide used to generate P9L was 3′-GAC GAA CCG CGA *CCC* CCG CGC CCC TTT-5′ and hybridizes to the adenylate kinase coding (+)-strand of single-stranded pEAK90 DNA, thus promoting a CCG to GGG codon change (Pro → Gly). This nucleotide exchange also leads to the loss of a *Nci*I restriction site (*Nci*I, CCGGG) in the adk gene and thus produces a different restriction pattern. From colonies obtained after the mutagenesis reaction, double-stranded DNA was prepared and analyzed by restriction digests with *Nci*I. Two out of eight colonies were positive, and from one of these dsDNA was prepared. The *Bst*EII–*Hin*dIII fragment was cut out of the mutant plasmid DNA and ligated into pEAK90 missing the corresponding fragment. The sequence of the resulting mutated pEAK plasmid was verified in the region of the mutated fragment by the method of Maxam and Gilbert (1980) using a solid support as described (Rosenthal et al., 1986). Generation of mutant plasmids for P9L, G10V, and K13Q was already described (Reinstein et al., 1988).

Protein Purification. The mutant protein P9G was prepared essentially as described (Reinstein et al., 1988) with a Blue Sepharose affinity column (Thompson et al., 1975) and a DEAE ion-exchange column. Since the mutant protein K13Q did not bind to Blue Sepharose, a modification of the protein preparation procedure was necessary. Flowthrough of the Blue Sepharose column was precipitated with 90% ammonium sulfate, dissolved in 20 mL of 50 mM Tris-HCl, pH 7.5, dialyzed twice against 2 L of 10 mM Tris-HCl, pH 7.5 at 4 °C, and loaded on a 2.5 × 20 cm Q-Sepharose fast-flow column (Pharmacia) and eluted with a linear gradient of 0–0.4 M KCl in 10 mM Tris-HCl, pH 7.5, and a total volume of 1 L at a flow rate of 400 mL/h. The mutant protein K13Q eluted at 0.12–0.16 M KCl. Purity was checked on native PAA gels.

Native Polyacrylamide Gels. Native discontinuous PAA gels were prepared as described by Blackshear (1984) with a pH of 6.8 for the stacking gel buffer, a pH of 8.8 for the resolving gel buffer, and a pH of 8.3 for the reservoir buffer. The final acrylamide concentration of the resolving gel was 15%.

Staining Procedures for Native PAA Gels. Staining with Coomassie Blue was performed as described for SDS-denaturing PAA gels by Laemmli (1970). A procedure for staining starch gels for the enzymatic activity of adenylate kinase has already been described (Kuhn & Jerchel, 1941; Fildes & Harris, 1966; Friedrich et al., 1984; Droese & Scheibe, 1987), and we used this procedure with slight modifications for activity staining of native PAA gels. This procedures couples the enzymatic reaction 2ADP → AMP + ATP to the production of a colored formazan compound. Components for staining solution were 100 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 2 mM ADP, 10 mM glucose, 0.5 mM

$NADP^+$, 0.05% (w/v) 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) as coloring compound, and 0.0125% (w/v) *N*-methylphenazinium methyl sulfate (PMS) as electron bridge. After the gels had been run, they were washed twice with 50 mM Tris-HCl, pH 7.5, and stained for about 20 min in 20 mL of activity staining solution with 20 units each of hexokinase and glucose-6-phosphate dehydrogenase (Boehringer Mannheim).

Preparation of mAP5A and mAP5Am. Preparations of fluorescent derivatives of the bisubstrate inhibitor AP5A were performed as described (Reinstein et al., 1990). Purity of the nonfluorescent nucleotides was controlled by HPLC as described in Reinstein et al. (1988).

Binding Studies with Fluorescent Analogues. Fluorescence measurements were performed with an SLM Smart 8000 photon-counting spectrofluorometer. Excitation wavelength was 360 nm and emission wavelength 440 nm. Unless otherwise indicated, the buffer was 50 mM Tris-HCl, pH 7.5 (25 °C), and the temperature of the cuvette was adjusted to 25 °C with a water bath. Affinities of fluorophore–enzyme (AK–mAP5Am) complexes and substrate–enzymes complexes were determined as described (Reinstein et al., 1990).

Steady-State Kinetics. To measure the kinetic parameters in the forward reaction (production of ADP), a coupled colorimetric assay was used (Berghäuser, 1975) which was optimized for adenylate kinase of *E. coli* (Reinstein et al., 1988). Protein concentrations were determined according to Bradford (1976) with BSA as standard. One unit of enzymatic activity in the direction of ADP production is defined as the amount of enzyme that catalyzes the chemical conversion of 1 μmol of AMP per minute. V_{max} and k_{cat} values of the variable substrate for the mutants could technically not be measured under saturating conditions of the fixed substrate, because of the low affinity of the substrates and the problems associated with high substrate concentrations (Reinstein et al., 1988). The measured values were corrected for full saturation as described before (Reinstein et al., 1988) with

$$V_{max,cor} = f_{cor} V_{max,obs}$$

$$k_{cat,cor} = f_{cor} k_{cat,obs}$$

$$f_{cor} = ([S_{fix}] + K_{mS_{fix}})/[S_{fix}]$$

Determination of Temperature Stability by Circular Dichroism. A fluorescence cuvette (0.4-cm diameter) was filled with 1.5 mL of 10 mM phosphate buffer (pH 7.5) containing 0.3 mg of protein. Circular dichroism was recorded with a Jobin-Yvon dichrograph Mark III at a fixed wavelength of 220 nm. The temperature increase per time was 40 °C/h with a Haake F3 waterbath. The temperature was measured within the cuvette. To determine the melting temperature, the following equation taken from Hecht et al. (1984), which is based on the van't Hoff relation, was used:

$$\theta_T = \theta_D + (\theta_N - \theta_D)/(1 + f)$$

where $f = \exp [\Delta H_{denat}/RT(T/T_m - 1)]$, θ_T is the ellipticity at temperature T (kelvin), θ_D is that of denatured protein, and θ_N is that of native protein, and T_m is the melting temperature (kelvin).

Determination of Temperature Stability by Fluorescence. Nearly equal amounts of fluorophore mAP5Am and purified enzyme were mixed at 25 °C in 1 mL of Tris-HCl buffer, pH 7.5 (25 °C) containing 2.5 mM EDTA and heated up to 70 °C in about 40 min. The fluorescence signal was observed and analyzed as described (Reinstein et al., 1990).

Preparation of NMR Probes. About 12–14 mg of protein was dialyzed twice against 20 mM potassium phosphate at 4 °C in the presence of mixed-bed ion-exchanger Amberlite ICR-718 (Serva). The resulting protein was freeze-dried, dissolved in 4 mL of D_2O (Merck) to exchange dissociable protons, and lyophilized again after 6 h at 4 °C. The freeze-dried protein was dissolved in 0.5 mL of D_2O (100%, Sigma) before measurement, and protein concentration was controlled according to the method of Ehresmann et al. (1973).

NMR Measurements. Samples were measured on a commercial Bruker AM-500 spectrometer with tubes of 5-mm diameter. The sample temperature was adjusted to 300 K with a precooled stream of dry air that was temperature regulated with a standard Bruker VT1000 unit. The residual HDO resonance was suppressed by selective irradiation at the HDO frequency. Chemical shifts of protons were referenced to internal DSS (sodium 4,4-dimethyl-4-silapentane-1-sulfonate).

Results

Preparation of Mutant Proteins. We have described earlier a very efficient expression system for adenylate kinase of *E. coli* where 30–40% of the soluble extract constitutes the enzyme adenylate kinase (Reinstein et al., 1988). In this system the *E. coli* adenylate kinase gene with its own promoter is cloned into the multicopy plasmid pEMBL. The plasmid-coded form of the enzyme constitutes approximately 99% of the total adenylate kinase of the extract. For the purification of highly active mutants of adenylate kinase like P9G, it is therefore not necessary to separate the plasmid-coded form of the enzyme from the chromosome-coded form because the contribution of wild-type enzyme to the properties of the purified enzyme is negligible. Thus, P9G was purified by the standard procedure as described [see also Reinstein et al. (1988)] without separation of wild-type from mutant enzyme.

By analysis of crude extracts from the system expressing K13Q, it became clear that this mutation is highly expressed but that it is either totally inactive or only slightly active. Thus, it was mandatory to separate the plasmid-coded mutant enzyme from chromosome-coded wild-type enzyme. This was accomplished by chromatography of the crude extract on Blue Sepharose, which binds wild-type enzyme but does not bind the mutant enzyme. Figure 2 shows the analysis of the purification of K13Q on a native polyacrylamide gel. One can see by Coomassie staining that the large amount of AK that does not bind to Blue Sepharose runs slightly different compared to wild-type enzyme on the native polyacrylamide gel (they do not separate on a SDS gel). We also analyzed the same gel by an activity staining procedure that couples adenylate kinase activity to NADH production and to a chemical reaction that produces a red dye (Fildes & Harris, 1966; Kuhn & Jerchel, 1941; see also Materials and Methods). One can clearly see that the Blue Sepharose separated a large amount of inactive (or slightly active; Figure 2, lanes B and C) enzyme from a small amount of very active enzyme (Figure 2, lanes D and E). Adenylate kinase from the flowthrough of the Blue Sepharose column was separated from other proteins by anion-exchange chromatography with Q-Sepharose. Analysis of the eluant with native PAA gel electrophoresis and the use of different staining methods indicates that the preparation of K13Q (Figure 2, lanes F and G) does not contain any wild-type enzyme (activity stain) and is free from other proteins (Coomassie stain).

Kinetic Analysis of Mutant Enzymes. Table I shows the steady-state analysis of wild-type and mutant enzymes. We have shown before that the replacement of Pro-9 and Gly-10 in the phosphate binding loop with aliphatic residues (P9L, G10V) leads to a drastic increase of the K_m values for substrates and does not greatly influence V_{max}. This was attributed

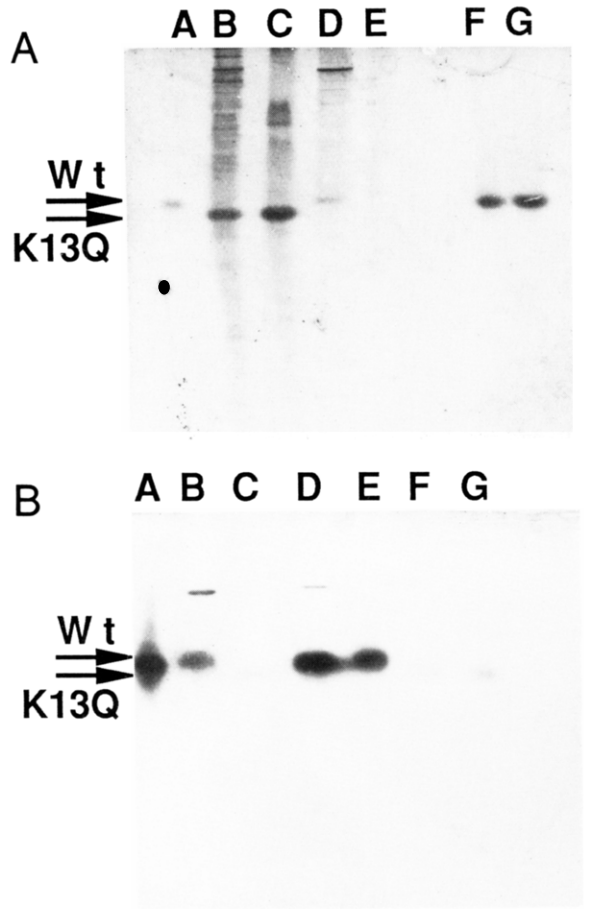

FIGURE 2: Analysis of a K13Q protein preparation using native polyacrylamide gel electrophoresis. Different probes as indicated below were run on native polyacrylamide gels as described under Materials and Methods. One gel was stained with Coomassie Blue (A) and the other was stained for adenylate kinase activity as described under Materials and Methods (B). The migrations of wild type and K13Q are slightly different and are indicated by arrows. Lanes: (A) 0.5 μg of AKec; (B) 1.5 μL of K13Q crude extract (from a total of 200 mL); (C) flowthrough Blue Sepharose; (D) KCl eluate (about 0.4 M) from Blue Sepharose (fraction 80); (E) KCl eluate (about 0.4 M) from Blue Sepharose (fraction 92); (F) KCl eluate (0.18 M) from Q-Sepharose column (fraction 52); (G) pooled KCl eluate, fractions 53–56.

to the induced-fit mechanism of adenylate kinase, which postulates that the enzyme has an active and an inactive conformation and that nearly all substrate-bound enzyme is in the active form. In the mutants the equilibrium between active and inactive enzyme is disturbed (Reinstein et al., 1988). It was interesting to find that the P9G mutation has the same effect of increasing the K_m values for ATP and AMP by a factor of 11 and 13.5, respectively. As in the loop mutants P9L and G10V described earlier, V_{max} is very similar to that of wildtype. Indeed, if corrections are made for incomplete saturation by the fixed substrate due to the high K_m, the V_{max} is increased by a factor of 2 as compared to that of wild type. With K13Q was also find a comparable increase in the K_m of substrates as in all the other mutants of the phosphate binding loop. Here, V_{max} and k_{cat} are however drastically decreased by a factor of 1.5×10^4 and the specificity constants k_{cat}/K_m by factors of 2.7×10^5 (ATP) and 4.5×10^5 (AMP), respectively.

Difference Energy Diagrams. A versatile and sensitive system for analyzing the use of binding energies along the reaction path was introduced by Wells and Fersht (1986). The changes in the free energy of binding of isolated substrates, combined substrates, or the transition state to the enzyme are

Table I: Steady-State Kinetic Constants of Wild-Type and Mutant Adenylate Kinases of *E. coli* at 25 °C[a]

enzyme	substrate	K_m (μM)	V_{max} (units/mg)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})
WT	ATP	71	**780**	**305**	**4.3**
			840	305	4.65
	AMP	26	**770**	**301**	**11.6**
			825	322	12.4
P9G	ATP	775	**890**	**348**	**0.449**
			1650	646	0.834
	AMP	350	**1100**	**430**	**1.23**
			2160	846	2.42
K13Q	ATP	1400	**0.043**	**0.016**	**0.000 012**
				0.06	0.023 6, 0.000 016
	AMP	825	**0.044**	**0.017**	**0.000 02**
			0.056	0.022	0.000 026

[a] The measured values (in boldface) for V_{max}, k_{cat}, and k_{cat}/K_m are extrapolated to saturation also for the site of the fixed substrate to give the corrected values (in lightface). This was done by use of the known K_m value of this substrate and the equations described under Materials and Methods and in Reinstein et al. (1988). Concentrations of fixed substrates were 300 μM AMP (WT and P9G) and 2000 μM AMP (K13Q) for the determination of K_{mATP}. The corresponding concentrations for the determination of K_{mAMP} were 800 μM ATP (WT and P9G) and 5000 μM ATP (K13Q).

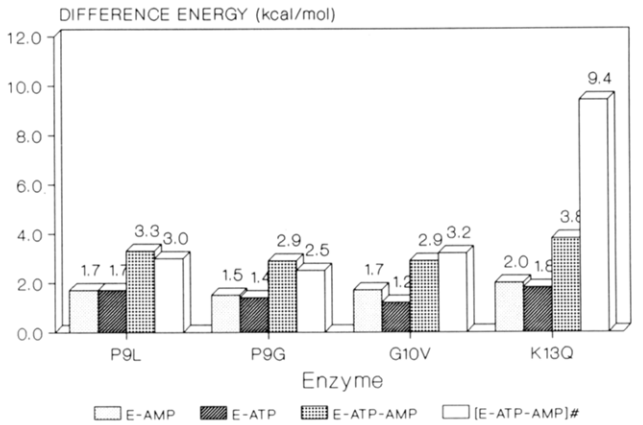

FIGURE 3: Difference energy diagrams for mutant adenylate kinases. Difference energy diagrams were calculated as described (Wells & Fersht, 1986; see also Results). Free energies of mutant enzyme–substrate complexes are subtracted from the free energies of the corresponding wild-type enzyme complexes .

calculated, and the differences to the corresponding wild-type values are plotted. The equations given by Wells and Fersht (1986) are

free energy for binding of one substrate (ATP or AMP)

$$\Delta G = RT \ln K_d(\text{AK–MgATP,AK–AMP})$$

free energy for combined binding of substrates

$$\Delta G = RT \ln [K_d(\text{AK–MgATP})K_d(\text{AK–AMP})]$$

free energy for the transition state

$$\Delta G^{\ddagger} = RT \ln (k_b T/h) - RT \ln [k_{cat}/K_d(\text{AK–AMP})K_d(\text{AK–MgATP})]$$

where k_b is the Boltzmann constant, h is Planck's constant, and k_{cat} is the overall reaction rate of the enzyme.

The difference energy for each binding step in the diagram is calculated as

$$\Delta\Delta G = \Delta G(\text{mutant complex}) - \Delta G(\text{WT complex})$$

The calculated difference energy diagrams are shown in Figure 3. The increase in difference energies for the binding of substrates is very similar for all mutations in the phosphate binding loop. The difference energy calculated for the transition state of K13Q, however, shows a pronounced shift of

Table II: Dissociation Constants (μM) of Fluorophores and Substrates of Adenylate Kinases and of Various Bisubstrate Inhibitors of Adenylate Kinases[a]

Part A

	mAP5Am		ATP					
enzyme	$MgCl_2$	EDTA	$MgCl_2$	EDTA	ADP, EDTA	AMP EDTA	GTP, $MgCl_2$	ATP K_m[b]
WT	0.3	0.16	85	35	4	520	1500	71
P9L	1.5	0.82	470	160	19	193	4650	1180[c]
P9G	1.43	0.79	655	160	24	567	3040	775
G10V	2.2	0.51	690	170	75	500		530[c]
K13Q	1.62	12	1410	960	318	770		1400

Part B

	AP4A		AP5A		AP6A	
enzyme	$MgCl_2$	EDTA	$MgCl_2$	EDTA	$MgCl_2$	EDTA
WT	43	13	0.015	0.1	0.108	0.157
P9L	148		0.29	0.57		
P9G	105		0.308	0.305		
G10V			0.056	1.4		
K13Q	146	165	0.037	58	0.122	14

[a] All fluorescence titration experiments were performed in 50 mM Tris-HCl, pH 7.5 and 25 °C, containing 2.5 mM EDTA or 5 mM $MgCl_2$ [for details, see Reinstein et al. (1990)]. [b] K_m values of ATP have been added for comparison. [c] These constants have been determined earlier (Reinstein et al., 1988).

9.4 kcal/mol compared to that of the wild-type enzyme. Since the difference energies for the simultaneous binding of both substrates equal those of the transition state for all mutant enzymes except K13Q, these other amino acids are not involved in stabilizing the transition state.

Dissociation Constants of Substrates and Analogues. In the preceding paper (Reinstein et al., 1990) we have shown that by use of a fluorescent analogue of the enzyme inhibitor AP5A the affinities of various substrates and inhibitors with and without Mg^{2+} can be conveniently measured. Information on these affinities may help us to monitor the structural changes that have been generated by the mutations and to compare the different mutant enzymes. To obtain a more detailed description of the effects of mutations in the phosphate binding loop, we used the mutants that we have described before, P9L and G10V (Reinstein et al., 1988), together with P9G and K13Q, described here.

Active site titrations with mAP5Am indicated that all proteins are at least 90% active in the binding of fluorophore. The results of fluorescence titrations are shown in Table II. The first row lists the affinities of wild-type and mutant proteins for the fluorescent analogue mAP5Am in the presence and absence of Mg^{2+}, measured directly by fluorescence titration as shown in the accompanying paper (Reinstein et al., 1990). The dissociation constants of mAP5Am for the mutants are increased as compared to those of wild type although the magnitude of this increase is smaller than for the K_d or K_m values of the substrates AMP and ATP. This suggests that the binding affinity of the fluorophore is dominated by hydrophobic interactions between protein and the mant group, which are not influenced by the mutations. The affinities of the proteins for ATP in the presence and absence of Mg^{2+} are measured by displacing the fluorophore from the protein or by competitive binding as shown before (Reinstein et al., 1990).

The dissociation constants for the enzyme–ATP interaction correspond very closely to the $K_{m,ATP}$ values found by the steady-state analysis except for P9L, where the steady-state kinetics yields a value of 1180 μM as compared to 470 μM for the K_d. Table IIA also shows that the affinity of AMP for the ATP site of P9L is much higher than for wild type and other mutant enzymes. Thus, in the determination of the K_m value for ATP a correction has to be made for the AMP binding to the ATP site:

$$K_{m,app} = K_m[1 + [AMP]/K_i(AK\text{–}AMP)]$$

where K_i is the dissociation (inhibition) constant of AMP for the ATP site. This correction is particularly severe for P9L with its high affinity of AMP for the ATP site (193 μM). This would explain the great discrepancy between K_M and K_d for the P9L–ATP interaction.

The fact that the dissociation constants equal the corresponding K_m values indicates that AKec obeys a rapid equilibrium mechanism and strongly supports our interpretation that the elevated K_m values of the loop mutants are a consequence of an altered equilibrium between active and inactive enzyme forms (Reinstein et al., 1988).

As described in the accompanying paper (Reinstein et al., 1990) the binding of AMP to its proper binding site (K_m value 26 μM) does not displace the fluorophore from the protein and is thus not measurable with the fluorescence technique. At higher concentrations AMP displaces the fluorophore from the ATP binding site, and the dissociation constants measured reflect the affinity of AMP for the ATP site and therefore are equal to the K_i values for AMP. This shows that the AMP binding site does not tolerate the modified ribose moiety of mAP5Am and confirms earlier results that it is very specific (Saint Girons et al., 1987).

In column 6 of Table IIA the affinities between enzyme and ADP in the absence of Mg^{2+} (which obviously would start the enzymatic reaction) are listed, and here, the K13Q mutant shows the biggest effect because it raises the dissociation constant by a factor of 80, which is by far the strongest effect on binding strength of any loop mutant.

AP5A has long been known to be a very potent inhibitor of adenylate kinase. It is believed to be a bisubstrate inhibitor (Lienhard & Secemski, 1973). Although ATP and AMP together have only four phosphate groups, their spatial separation seems to be matched by the five phosphate groups of AP5A, while the separation is too small and too large in AP4A and AP6A, respectively. To get more quantitative data on the binding of bisubstrate analogues, we investigated and compared the binding properties of AP4A, AP5A, and AP6A by displacing the fluorophore mAP5Am from the protein. These data are shown in Table IIB. The strength of binding of the three inhibitors to adenylate kinase is in the order AP5A > AP6A > AP4A, and it is noteworthy that AP6A is a still better inhibitor than AP4A. This order is the same for wild-type and, where it has been investigated, for mutant proteins. The most notable difference between wild-type and mutant protein is found for K13Q: The difference in affinity for the binding

Table III: Temperature Stabilities of Adenylate Kinases[a]

	fluorescence method		CD method	
enzyme	t_m (°C)	ΔH_{denat} (kJ/mol)	t_m (°C)	ΔH_{denat} (kJ/mol)
WT	55.6	425	54	472
P9G	49	355		
P9L	49.8	450		
G10V	49.5	275		
K13Q	52.5	298	51.8	447

[a] Temperature stabilities of wild-type and mutant proteins of AKec measured with two different methods (see Results). ΔH_{denat} indicates the standard enthalpy of denaturation and t_m the melting point (the temperature where 50% of the protein is denatured).

of AP5A in the presence and in the absence of Mg^{2+} is 1600-fold for K13Q and only 6-fold for wild-type enzyme. For the other mutants the difference in affinities varies between 0 and 3.

Thermal Stability of Proteins. To investigate the effect of the mutations on the stability of adenylate kinase, we measured the thermal stabilities of wild-type and mutant proteins by the fluorescence technique described by Reinstein et al. (1990). The results are shown in Table III. We have shown before (Haase et al., 1989) that, by measuring the fluorescence of protein-bound fluorophore with increasing temperature, denaturation of the protein can be monitored. The melting temperatures found by this method correspond very closely to the melting temperatures as determined by CD. Using this technique for the mutant proteins, we find that melting temperatures of P9L, P9G, and G10V are very close to each other (49–49.8 °C) and 6 deg lower than that of wild type. K13Q is more heat stable than the other loop mutants with a melting temperature of 52.5 °C, 3 deg less than that of wild-type protein.

Structural Investigation by NMR. Adenylate kinase has been investigated extensively by NMR, and the spectrum of the protein is exceptionally well suited for a detailed investigation because a great number of individual resonances can be identified. Some sequence-specific assignments have been made already (Bock et al., 1988). We use NMR to investigate whether site-specific mutations change the conformations of the protein. The aromatic portion of the spectra of wild-type and mutant proteins is shown in Figure 4. One can see immediately that all the proteins have a discrete three-dimensional structure with many of the aromatic protons well resolved. It also shows that the mutant proteins with amino acid substitutions in the phosphate binding loop have a spectrum that is different from that of wild type. Although it is difficult to follow the change in chemical shift for a particular proton resonance, especially in a one-dimensional spectrum, we have indicated in Figure 4 the shift of the resonances for His_B and His_C and for Tyr-181. These can be most easily followed because they are separated from the others. These resonances have been assigned earlier by Bock et al. (1988). We have reported before that the G10V and the P9L mutations change the structure of the protein. As shown here, the P9G mutation also changes the structure of the protein, and the change is different from the P9L mutation. The spectra also indicate that K13Q has a well-defined structure that is again different from that of wild type and from the structure of the other mutants. It seems however from the 1-D NMR spectrum that K13Q is more similar in structure to the wild-type enzyme than are the other loop mutants.

Since KQ13 has a drastically reduced catalytic activity, it would be important to study in detail the structure of the protein–nucleotide complex. For such studies it would be necessary to use 2-D NMR techniques. In preliminary NOESY experiments we found however that AP5A is bound similarly in wild-type and KQ13 enzyme (Vetter et al., unpublished results).

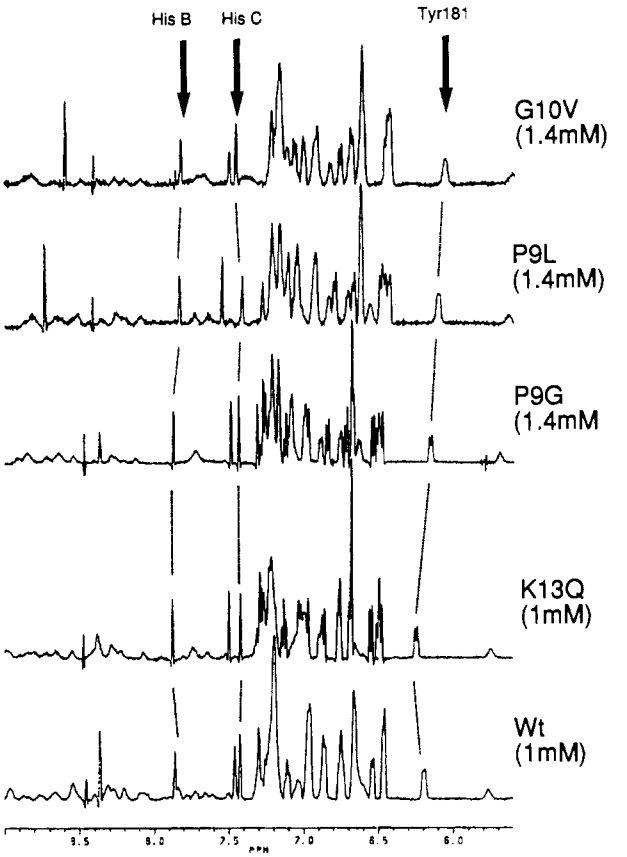

FIGURE 4: Proton NMR spectra of the aromatic side chains of *E. coli* adenylate kinase wild-type and mutant proteins. The concentrations of the individual samples are indicated, and certain resonances are marked throughout the different spectra. Experimental conditions: temperature, 300 K; buffer, 40–100 mM potassium phosphate. An internal DSS (sodium 4,4-dimethyl-4-silapentane-1-sulfonate) reference was used for calculation of chemical shifts in ppm. All spectra were digitally processed by Gaussian line sharpening.

Discussion

It has been suggested that the affinity dye Cibacron Blue binds to a supersecondary structural motif, the "nucleotide fold" of mono- and/or dinucleotide binding enzymes and proteins (Thompson et al., 1975). Further investigations, especially with lactate dehydrogenase and phosphoglycerate kinase, proved that this affinity dye is a competitive ligand with respect to natural substrates like NAD/NADH or ATP/3-phosphoglycerate (PGA), respectively (Thompson & Stellwagen, 1976). Comparisons of UV spectra of poly(L-lysine) and poly(L-arginine) complexed with Cibacron Blue to different protein-Cibacron Blue complexes indicated that the interactions between dye and protein are mainly due to ionic interactions between sulfate groups of the dye and arginine or lysine amino acid side chains of the protein (Subramanian, 1982). Adenylate kinases bound to Blue Sepharose columns are eluted very specifically by substrates and/or inhibitors (Feldhaus et al., 1975; Bârzu & Michelson, 1983). We have described two mutant proteins of AKec, K13Q (this paper) and R88G (Reinstein et al., 1989), which are not able to bind to Blue Sepharose columns. Lys-13 and Arg-88 are both situated very close together in the active site of AKec (Müller & Schulz, 1988; Egner et al., 1987). It is possible

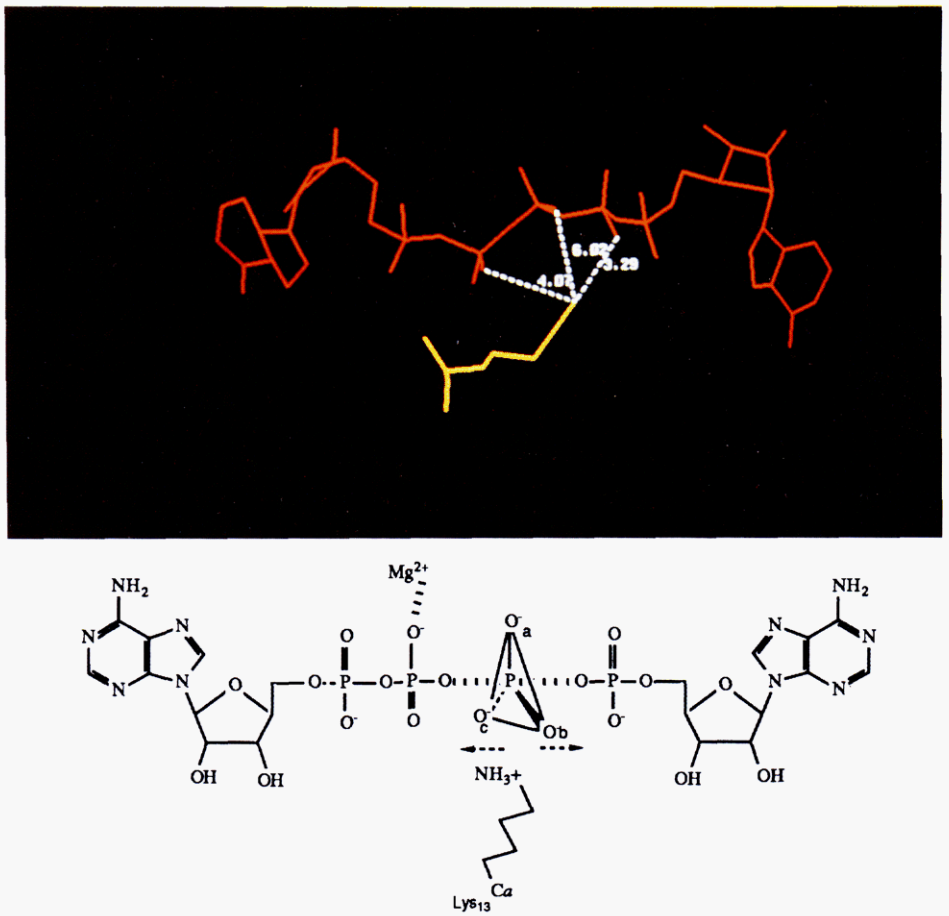

FIGURE 5: Interaction of Lys-13 with phosphate groups of AP5A. (Top) Distances of Lys-13 to the nearest oxygen atoms of phosphates P2, P3, and P4 in the complex of AKec with AP5A (Müller & Schulz, 1988; Schulz et al., unpublished results) as determined by X-ray crystallography. Distances of phosphate oxygen atoms OP2R, OP3R, and OP4S (4.07, 6.02, and 3.29 Å, respectively) are indicated by white labels. The distances of the corresponding lysine (Lys-30 using the numbering of AKy) in the AKy–AP5A complex are reported to be 0.34 Å for OP2R and 0.36 Å for OP4S (Egner, 1986; Egner et al., 1987). Coordinates of the X-ray structure were a generous gift from G. E. Schulz, Freiburg, FRG. (Bottom) Sketch for the proposed role of Lys-13 in stabilizing the pentacovalent transition state of the transferred phosphoryl group in the catalytic reaction.

that that the lack of binding capacities in both mutant proteins is due to the loss of ionic interactions with the same sulfate group of the Cibacron Blue dye.

As outlined in the introduction, the phosphate binding loop of a number of guanine and adenine binding proteins has the Walker consensus sequence G-X-*Z*-X-X-G-K-S/T, where *Z* is often glycine or proline (Walker et al., 1982). The three-dimensional structures of the p21–GTP complex (Pai et al., 1989) and the AK–AP5A complexes with AK from two different organisms (Egner et al., 1987; Müller & Schulz, 1988; Schulz et al., unpublished results) have recently been determined. They show that the peptide chain structure of the first 20 or so amino acids of the two proteins and the relative locations of GTP and the ATP portion of AP5A can be aligned and are virtually identical (Vetter et al., 1989). In this loop the position of amino acid *Z* is Gly-12 in all p21 proteins and Pro-9 in all adenylate kinases. Mutations of this amino acid in p21 to any other amino acid except proline make the protein oncogenic (Seeburg et al., 1984). The mutation of this glycine to proline however, although not oncogenic, changes the properties of the protein such that the GTPase is increased and no longer responsive to the GTPase activating protein GAP (Gibbs et al., 1988).

We have shown before that the mutation in AKec of Pro-9, which corresponds to Gly-12 in p21, to leucine changes the K_m values of the substrates but does not decrease the rate of the enzymatic reaction. We show here that the mutation of this residue to glycine also changes the structure and substrate binding properties (K_m) of adenylate kinase. As with the other loop mutants, P9L and G10V, the catalytic rate (k_{cat}) of the enzymatic process is not decreased. It might even have a higher catalytic activity if full saturation of the substrate binding sites could be achieved. We would have expected that the exchange of glycine by proline in p21 and the exchange of proline by glycine here would not have any effect on the structure or activity. One has to conclude that the structural integrity of the phosphate binding loop is also influenced by the other amino acids in the loop except G, *Z* (Gly or Pro), and K (Lys) and it is these amino acids X, usually small aliphatic residues, that are not conserved between the different classes of proteins.

The structural homology between the two proteins p21 and AK in the phosphate binding loop can be extended further. In the structures of both p21–GTP and AK–AP5A the conserved lysine residue is found to stabilize the tight structure of the loop by making contact to two main-chain peptide carbonyl atoms, which are residues 10 and 11 in p21 (Pai et al., 1989) and residues 7 and 8 in AKec (Dreusicke & Schulz, 1988). This interaction makes the loop appear as a circle of atoms around the β- and γ-phosphates. The lysine is also in a position to make contact to the γ-phosphate and to the β-phosphate oxygens in p21. Figure 5 (top) shows that, in the structure of AKec with the bisubstrate inhibitor AP5A, Lys-13 is in a position to make contact to the second and fourth but

not the third phosphate oxygens.

The proton NMR spectrum and the temperature-stability studies show that the structure of K13Q is different from that of wild type. The nucleotide binding data show that the local structure is sufficiently perturbed to weaken the interaction between substrates and enzyme. This effect is similar to that of other mutations in the loop that we and others (Reinstein et al., 1988; Tagaya et al., 1989) have described. We have shown that this decreased affinity does not itself decrease the rate of catalysis of adenylate kinase and may even increase it. The fact that K13Q is only weakly active suggests that the lysine residue is directly involved in catalysis. The energy profile of the mutant enzymes (Figure 3) shows that the K13Q mutation leads to a large decrease in the binding of the transition state only. Thus, we propose as a working hypothesis that the main effect of the lysine side chain is to stabilize the transition state as shown schematically in Figure 5 (bottom).

The structure of adenylate kinase with one or both substrates bound is not known at present. The structure of the complex between the inhibitor AP5A and AKec as shown in Figure 1 and 5 (top) demonstrates however that the lysine residue in the phosphate binding loop would be close enough to bind the β-phosphates of both ADP molecules represented by the second and fourth phosphates of AP5A.

A switch between two conformations of adenylate kinase of porcine muscle on the basis of two different crystal forms was described by Sachsenheimer and Schulz (1977) and in more detail with refined coordinates by Dreusicke and Schulz (1988). These two conformations were regarded by the authors as snapshots of a rather open (equivalent to substrate-free form = B-form) and a rather closed (substrate-bound form = A-form) conformation. Lys-21 (=Lys-13, AKec) of AK1 experiences the most pronounced structural change by shifting 8 Å in going from the A-form (substrate-bound form) to the B-form (open form). In the A-form this lysine points into the enzyme and makes contact to a sulfate ion and the main-chain nitrogens of Gly-15 and Gly-16 (numbering of AK1). We conclude that the phosphate binding loop of AK is responsible for mediating structural rearrangements upon binding substrates. Furthermore, it carries a lysine residue that may be very important in stabilizing the transition state. This situation may be compared to that of tyrosyl-tRNA synthetase, where also a mobile loop envelopes the transition state in an induced-fit mechanism (Fersht et al., 1988).

Since Lys-13 is in an appropriate position for effective catalysis of phosphoryl transfer only when *both* substrates [or presumably AP5A; see also Vetter et al. (1989)] are present (A-form), unwanted hydrolysis of ATP involving phosphoryl transfer to water as a side reaction is largely prevented. The ATP-AMP phosphoryl transfer reaction of K13Q is indeed only 10 times faster than its hydrolysis rate (data not shown); it is 100000 times faster in the wild-type enzyme. The absolute value for the ATP hydrolysis rate is the same for both wild-type and K13Q protein.

Considering the degree of conservation of the lysine residue in the phosphate binding loop of nucleotide binding proteins and the conservation of the three-dimensional structure of this loop, it will be interesting to see if its functional role is identical in the different proteins.

ACKNOWLEDGMENTS

We thank R. S. Goody for the gift of mAP5Am and helpful discussions, E. Schiltz for sequencing the N-terminus of P9L and P9G, Marija Isakov for excellent technical assistance, G. E. Schulz for generously providing us with the coordinates of the structure of the AKec–AP5A complex, and K. C. Holmes for continuous support.

Registry No. AK, 9013-02-9; L-Lys, 56-87-1; L-Pro, 147-85-3; Gly, 56-40-6; L-Gln, 56-85-9; L-Leu, 61-90-5; L-Val, 72-18-4; ATP, 56-65-5; AMP, 61-19-8; ADP, 58-64-0; GTP, 86-01-1; mAP5Am, 128053-72-5; AP4A, 5542-28-9; AP5A, 41708-91-2; AP6A, 56983-23-4.

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Complexes of *Escherichia coli* Adenylate Kinase and Nucleotides: ^{1}H NMR Studies of the Nucleotide Sites in Solution

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Received August 16, 1989; Revised Manuscript Received April 2, 1990

ABSTRACT: One- and two-dimensional nuclear magnetic resonance (NMR) studies, in particular substrate–protein nuclear Overhauser effect (NOESY) measurements, as well as nucleotide and P^1,P^5-bis(5′-adenosyl) pentaphosphate (AP_5A) titrations and studies of the temperature-dependent unfolding of the tertiary structure of *Escherichia coli* adenylate kinase (AK_{EC}) were performed. These experiments and comparison with the same type of experiments performed with the porcine enzyme [Rösch, P., Klaus, W., Auer, M., & Goody, R. S. (1989) *Biochemistry 28*, 4318–4325] led us to the following conclusions: (1) At pH 8 and concentrations of approximately 2.5–3 mM, AK_{EC} is partially unfolded at 318 K. (2) ATP·Mg^{2+} binds to the ATP site with a dissociation constant of approximately 40 μM under the assumption that ATP binds to one nucleotide site only. (3) AP_5A·Mg^{2+} binds to both nucleotide sites and thus simulates the active complex. (4) The ATP·Mg^{2+} adenine in the AK_{EC}·AP_5A·Mg^{2+} complex is located close to His^{134} and Phe^{19}. (5) The AK_{EC} "G-loop" with bound ATP·Mg^{2+} is structurally highly homologous to the loop region in the oncogene product p21 with bound GTP·Mg^{2+}.

Adenylate kinases (AK,[1] ATP:AMP phosphotransferase, EC 2.7.4.3) catalyze the transfer of the terminal phosphoryl group of ATP to AMP in the presence of a divalent metal ion, physiologically Mg^{2+}, which binds to the triphosphate and one of the product diphosphates, respectively (Noda, 1971; Ray et al., 1988):

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

According to the reaction scheme, the enzyme has two different nucleotide binding sites, the AMP site and the ATP·Mg^{2+} site, corresponding to the product ADP and ADP·Mg^{2+} site, respectively. The location of these two sites is a subject of much controversy between X-ray crystallographers (Pai et al., 1977; Egner et al., 1987; Müller & Schulz, 1988) and NMR spectroscopists (Hamada et al., 1979; Smith & Mildvan, 1982; Fry et al., 1985; Mildvan, 1989).

The bisubstrate analogue P^1,P^5-bis(5′-adenosyl) pentaphosphate (AP_5A) is a potent inhibitor of all tested adenylate kinases, in particular of the porcine enzyme (Lienhard & Secemski, 1973; Feldhaus et al., 1975). Whereas it was not possible so far to cocrystallize any of the mammalian adenylate kinases with either one of the substrates or, alternatively, the

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[1] Abbreviations: ADP, adenosine diphosphate; AK, adenylate kinase; AK_1, cytosolic adenylate kinase; AK_{EC}, *E. coli* adenylate kinase; AK_Y, yeast adenylate kinase; AMP, adenosine monophosphate; AP_5A, P^1,P^5-bis(5′-adenosyl) pentaphosphate; ATP, adenosine triphosphate; COSY, correlated spectroscopy; 1D, one dimensional; 2D, two dimensional; δ_{AH} and δ_A-, chemical shift at low and high pH, respectively; δ_f and δ_b, chemical shift in the free and bound form, respectively; DQF-COSY, double quantum filtered COSY; DSS, sodium 2,2-dimethyl-2-silapentanesulfonate; DTE, dithioerythritol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-pressure liquid chromatography; K_d, dissociation constant; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy.

0006-2960/90/0429-7459$02.50/0