Structural and catalytic role of arginine 88 in Escherichia coli adenylate kinase as evidenced by chemical modification and site-directed mutagenesis

Reinstein, Jochen; Gilles, Anne-Marie; Rose, Thierry; Wittinghofer, Alfred; Girons, Isabelle Saint; Bârzu, Octavian; Surewicz, Witold K.; Mantsch, Henry H.

Publisher's version / Version de l'éditeur:
https://doi.org/10.1016/S0021-9258(18)83156-8
The Journal of Biological Chemistry, 264, 14, pp. 8107-8112, 1989-05-15

THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 264, No. 14, Issue of May 15, pp. 8107-8112, 1989
Printed in U.S.A.

Structural and Catalytic Role of Arginine 88 in *Escherichia coli* Adenylate Kinase as Evidenced by Chemical Modification and Site-directed Mutagenesis*

(Received for publication, September 16, 1988)

Jochen Reinstein‡, Anne-Marie Gilles§, Thierry Rose§, Alfred Wittinghofer‡, Isabelle Saint Girons§, Octavian Bârzu§¶, Witold K. Surewicz‖, and Henry H. Mantsch‖

From the ‡Max-Planck Institut für Medizinische Forschung, Abteilung Biophysik, 6900 Heidelberg, West Germany, §Institut Pasteur, Département de Biochimie et Génétique Moléculaire, 75724 Paris, France, and ‖National Research Council of Canada, Molecular Spectroscopy Section, Ottawa K1A OR6, Canada

Phenylglyoxal inactivates *Escherichia coli* adenylate kinase by modifying a single arginine residue (Arg-88). ATP, ADP, P^1,P^5-di(adenosine 5')-pentaphosphate, and to a lesser extent AMP protect the enzyme against inactivation by phenylglyoxal. Site-directed mutagenesis of Arg-88 to glycine yields a modified form of adenylate kinase (RG88 mutant) closely related structurally to the wild-type protein as indicated by Fourier transform infrared spectroscopy, differential scanning calorimetry, and limited proteolysis. However, this modified protein has only 1% of the maximum catalytic activity of the wild-type enzyme and 5- and 85-fold higher apparent K_m values for ATP and AMP, respectively, than the parent adenylate kinase. Arg-88, which is a highly conserved residue in all known molecular forms of adenylate kinases (corresponding to Arg-97 in muscle cytosolic enzyme), should be located inside a big cleft of the molecule, close to the phosphate-binding loop. It possibly stabilizes the transferable γ-phosphate group from ATP to AMP in the transition state.

ATP-dependent enzymes represent a large group of catalysts that use the nucleotide for cyclization reactions or as donor of phosphate, nucleotidyl, or adenosyl groups to suitable acceptors.

Knowledge of the primary structure and information derived from x-ray studies (1–4) makes it possible to localize the ATP-binding site in many of these proteins. Adenylate kinase represents a most suitable model for such studies for several reasons. It is relatively small (between 194 and 238 residues depending on the species) and has been well conserved throughout evolution (5). The catalytic site of adenylate kinase has two distinct nucleotide-binding sites: one interacting with MgATP or MgADP (the donor site), the other interacting with metal-free ADP or AMP (the acceptor site). Despite efforts to describe these sites unequivocally using a large variety of techniques including x-ray crystallography and NMR spectroscopy, ambiguities still persist regarding the location of the ATP and AMP sites in the three-dimensional structure of the protein (6–13).

Hamada *et al.* (14) isolated a peptide from rabbit muscle adenylate kinase which corresponds to residues 1–44 (MT-I) of the protein and which is able to bind εATP, a fluorescent analogue of ATP. Extensive NMR studies by Fry *et al.* (11–13) of a synthetic peptide 1 residue longer than MT-I suggested that it represents most of the MgATP-binding site of the mammalian enzyme. In fact, adenosine diphosphopyridoxal inactivated rabbit muscle adenylate kinase by reacting with Lys-21 (15). This residue belongs to a large flexible glycine-rich loop. The inactivation of adenylate kinase by adenosine diphosphopyridoxal was confirmed and extended by site-directed mutagenesis of an equivalent lysine residue in *Escherichia coli* adenylate kinase (Lys-13) and of other residues situated in the glycine-rich loop (16).

Another residue shown to be essential for nucleotide binding in various kinases and other ATP-dependent enzymes is arginine (17–21). In this report, we show that chemical modification of a single arginine residue in *E. coli* adenylate kinase (Arg-88) by phenylglyoxal results in enzyme inactivation. Site-directed mutagenesis of Arg-88 with glycine (RG88 mutant)[1] confirmed the essential role of this positively charged residue for both nucleotide binding and catalysis.

EXPERIMENTAL PROCEDURES

Chemicals—Adenine nucleotides, restriction enzymes, T4 DNA ligase, and coupling enzymes were from Boehringer, Mannheim. DNA polymerase large fragment (Klenow) was from Du Pont-New England Nuclear. TPCK-treated trypsin and soybean trypsin inhibitor were from Sigma. Phenylglyoxal monohydrate was from Aldrich. Collagenase from *Achromobacter iophagus* was purified according to Lecroisey *et al.* (22). Blue-Sepharose, polybuffer exchanger 94, and polybuffer 74 were all from Pharmacia LKB Biotechnologies Inc. mAp_5Am, a fluorescent derivative of Ap_5A with two *N*-methyl anthraniloyl groups coupled to the hydroxyl groups of the riboses via an ester linkage, was kindly provided by R. S. Goody (Max Planck Institut für Medizinische Forschung, Heidelberg, West Germany). Oligonucleotides were synthesized according to the phosphoamidinate method using a commercial DNA synthesizer (Cyclone™ Biosearch). [α-^{32}P]ATP (3000 Ci/mmol) was purchased from the Radiochemical Centre, Amersham Corp., and phenyl[2-^{14}C]glyoxal (30 Ci/mmol) was obtained from the Centre d'Etudes Nucléaires de Saclay (Gil-Sur-Yvette, France).

* This work was supported by Grant U.A. 1129 from the Centre National de la Recherche Scientifique, by a grant from the Ministère de la Recherche et de l'Enseignement Supérieur (France), and by the Canada-France Science and Technology cooperation program. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed.

[1] The abbreviations used are: RG88, the mutant whose glycine replaces Arg-88; Blue-Sepharose, Cibacron Blue 3G-A Sepharose CL-6B; Ap_5A, P^1,P^5-di(adenosine 5')-pentaphosphate; mAp_5Am, a fluorescent derivative of Ap_5A; HPLC, high performance liquid chromatography; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

Bacterial Strains and Plasmids—The temperature-sensitive *E. coli* K12 strain CR341T28 is from the Pasteur Institute collection. GT836 is CR341T28 carrying the pIPD37 recombinant plasmid, overproducing wild-type adenylate kinase (23). The *E. coli* K12 strain SMH50 *Δlac-pro, ara, thi, F'traD36, proAB, lacI*^{q}, *lacZΔM15* has been described earlier (24). Plasmids pEAK90 and pAK601 have been described earlier (16, 25).

Cloning Methods—Enzyme buffers were used, and transformation procedures were carried out according to Maniatis *et al.* (26).

Site-directed Mutagenesis—Site-directed mutagenesis was carried out with the pEAK90 recombinant plasmid carrying the *adk* gene in the pEMBL9 vector which can be induced to produce single-stranded DNA (16). Mutation was done using the method of Taylor *et al.* (27). The sequence of the oligonucleotide used to change the CGU codon (Arg-88) to GGU (Gly-88) is as follows: 3′ CG AAG GGC CCA TGG TAA 5′.

Purification of Adenylate Kinase and Activity Assays—The wild-type enzyme from the adenylate kinase-overproducing strain GT836 of *E. coli* was purified as described previously (23, 28). Adenylate kinase of the mutant strain (RG88) was purified in a three-step procedure involving chromatofocusing (Fig. 1), gel permeation, and Blue-Sepharose chromatography (23, 28). Blue-Sepharose chromatography was necessary to remove a low proportion of the wild-type enzyme encoded by the chromosome and is based on the fact that adenylate kinase RG88 does not bind to Blue-Sepharose. Adenylate kinase activity was determined at 340 nm and 25 °C in 1 ml final volume on a Beckman DU-7 computing spectrophotometer or a UV-260 Shimadzu spectrophotometer. One unit of enzyme activity corresponds to 1 μmol of product formed/min.

Chemical Modification—Adenylate kinase (4 mg/ml) in 50 mM potassium borate (pH 8) was incubated at 20 °C with various concentrations (between 1 and 10 mM) of phenylglyoxal. Samples of 5 μl, removed at different times, were diluted in 2 ml of the same buffer to stop the modification reaction before being assayed for activity. For determination of the amount of bound phenylglyoxal, the enzyme was treated with phenyl[2-^{14}C]glyoxal (3000 cpm/nmol). At various times, 0.1–0.5-ml samples of modified enzyme were separated from excess radioactive phenylglyoxal by gel filtration through a Sephadex G-25 column (1 × 10 cm) equilibrated with 50 mM potassium borate (pH 8) or 50 mM ammonium bicarbonate (pH 7.8), then immediately assayed for enzyme activity and radioactivity. Samples in ammonium bicarbonate were lyophilized.

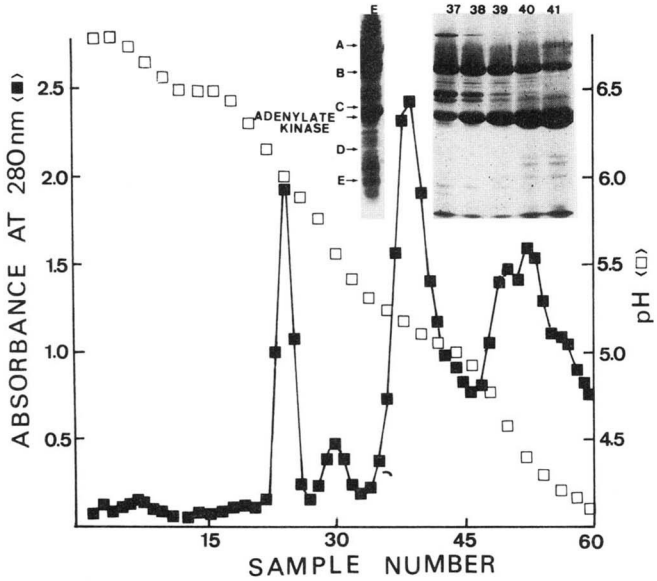

FIG. 1. **Chromatofocusing of the *E. coli* extract containing overexpressed RG88 mutant adenylate kinase.** The bacterial extracts were loaded onto a polybuffer exchanger 94 column (1.2 × 40 cm) at a ratio of 20 mg of protein/ml of swollen gel. After washing with 250 ml of 20 mM imidazole (pH 7), proteins were eluted with 500 ml of 10-fold diluted polybuffer 74 adjusted to pH 4 with 1 N HCl. Samples of 4 ml were collected at a flow rate of 40 ml/h. The *inset* shows sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5%) of the bacterial extract (*E*) and of samples 37–41. The molecular weight markers from *top* to *bottom* (*arrows* on the *left* side of *lane E*) were: bovine serum albumin (67,000); ovalbumin (43,000); carbonic anhydrase (30,000); soybean trypsin inhibitor (20,100), and lyzozyme (14,400).

Enzymatic Digestion of [^{14}C]Phenylglyoxal-modified Adenylate Kinase and Purification of Labeled Peptides—About 1.5 mg of [^{14}C] phenylglyoxal-treated adenylate kinase (inactivation between 70 and 80%) was dissolved in 400 μl of 50 mM ammonium bicarbonate containing 30 μg of TPCK-treated trypsin and incubated for 1 h at 25 °C. Then 30 μg of α-chymotrypsin was added to the solution, and the digestion was allowed to continue for an additional 2 h. The reaction was stopped with acetic acid, then the digest was lyophilized. Peptides were purified by reverse-phase HPLC (Du Pont-New England Nuclear liquid chromatograph 8800) using a Nucleosil C-18 column (5 μm, 4.6 × 250 mm) and an ammonium acetate (pH 6)/acetonitrile elution system (29) at a flow rate of 1.5 ml/min and OD recording at 230 nm. Fractions were lyophilized, dissolved in 0.1 N HCl, and transferred to hydrolysis or sequencing tubes.

Trypsin Digestion and Peptide Separation of RG88 Mutant Adenylate Kinase—Adenylate kinase RG88 mutant protein in 50 mM ammonium bicarbonate (1 mg/ml) was digested at 37 °C for 16 h with TPCK-treated trypsin (1%, w/w). Purification of tryptic peptides was achieved by HPLC as described above.

Amino Acid and Sequence Analysis—Amino acid analyses were performed on a Biotronik amino acid analyzer LC 5001 after 6 N HCl hydrolysis for 22 h at 110 °C. Amino acid sequence was determined using the manual 4-dimethylaminoazobenzene 4′-isothiocyanate/phenyl isothiocyanate double-coupling technique. The resulting derivatives were then identified on polyamide layers as described by Chang *et al.* (30).

Nucleotide-binding Studies—Binding of ATP to *E. coli* adenylate kinase (wild type strain and RG88 mutant) was investigated by equilibrium dialysis (23) or by determining the fluorescence enhancement of mAp_5Am upon binding to enzyme in the absence or in the presence of ATP. Fluorescence measurements (excitation wavelength 360 nm, emission wavelength 440 nm) were done with an SLM "Smart" 8000 photon-counting spectrofluorimeter. For determination of the ATP-binding constant, 15 μM protein solution together with 1.12 μM mAp_5Am in 50 mM Tris-HCl (pH 7.5) and 10 mM $MgCl_2$ were titrated with increasing concentrations of ATP. The calculated fluorescence for infinite ATP concentration equals that of the free fluorophor, so ATP displaces fully the fluorescent analogue and is regarded as competitive with respect to fluorophor. The dissociation constant of the adenylate kinase/mAp_5Am complex was determined from the fluorescence titration of each particular protein with the nucleotide analogue and was used to calculate the dissociation constant for ATP.

Fourier Transform Infrared Spectroscopy—Infrared spectra were recorded at 22 °C with a Digilab FTS-60 spectrometer using a high sensitivity deuterated triglycine sulfate detector. Samples were prepared in 50 mM HEPES buffer in D_2O (pH 7.4) at a protein concentration of 1.3 mM, and the spectra were obtained as described previously (31).

Thermal Stability Experiments—The differential scanning calorimetry experiments were performed with a high sensitivity microcalorimeter (Microcal MC-2D) at a scanning rate of 1 K min^{-1}. The protein concentration varied between 1.4 and 1.6 mg/ml in a solution containing 50 mM HEPES (pH 7.4).

RESULTS

Kinetics of Adenylate Kinase Inactivation by Phenylglyoxal and Identification of Arg-88 as the Site of Chemical Modification—Incubation of *E. coli* adenylate kinase with phenylglyoxal in 50 mM borate (pH 8) resulted in progressive inactivation of the enzyme. The kinetics of inactivation is a first order process. Rate constants (k_{obs}) were dependent upon the concentration of phenylglyoxal (Table I). A plot of the reciprocals of k_{obs} *versus* the reciprocals of the concentration of phenylglyoxal allowed determination of the apparent K_{inact} for phenylglyoxal (9.4 mM) and of the maximum rate constant of inactivation of *E. coli* adenylate kinase at a saturating concentration of reagent (1.7×10^{-3} s^{-1}). ATP, ADP, Ap_5A, and AMP (to a lesser extent) protected adenylate kinase against inactivation by phenylglyoxal (Table I). It is interesting to note that porcine heart adenylate kinase, which is another

TABLE I

Inactivation of E. coli adenylate kinase by phenylglyoxal (PG) and protection by adenine nucleotides

Experimental conditions	Inactivation rate constant
	$s^{-1} \times 10^4$
1 mM PG	1.7
2 mM PG	3.1
4 mM PG	5.5
10 mM PG	9.1
4 mM PG + 10 mM $MgCl_2$	5.3
4 mM PG + 4 mM ATP	<0.2
4 mM PG + 4 mM ATP + 10 mM $MgCl_2$	<0.2
4 mM PG + 4 mM ADP	<0.2
4 mM PG + 4 mM ADP + 10 mM $MgCl_2$	<0.2
4 mM PG + 1 mM Ap_5A	<0.2
4 mM PG + 4 mM AMP	3.8
4 mM PG + 4 mM AMP + 10 mM $MgCl_2$	2.6

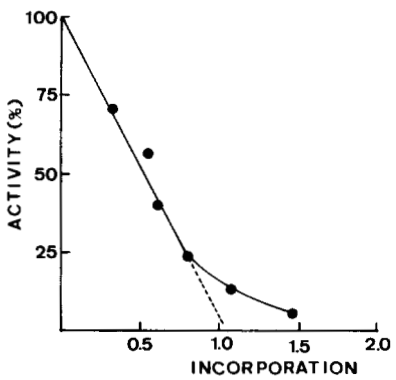

FIG. 2. **Correlation of inactivation of adenylate kinase with arginine modification by phenylglyoxal.** The mol of phenyl[2-^{14}C]glyoxal incorporated are given per mol of adenylate kinase (assuming a molecular weight of 23,500).

member of AK1 family, was inactivated by phenylglyoxal under more drastic conditions (19, 20).

Experiments with phenyl[2-^{14}C]phenylglyoxal demonstrated that up to 75% inactivation extrapolated to complete inactivation is correlated with the modification of 1 arginyl residue/molecule of enzyme (Fig. 2). The instability of phenylglyoxal arginine residues in proteins places limitations on the steps that can be used to identify the arginyl residues modified by phenylglyoxal. The best results were obtained when the ^{14}C-treated protein was digested in ammonium bicarbonate at 25 °C with 2% (w/w) trypsin for 1 h then with 2% (w/w) chymotrypsin for 2 h. HPLC separation of released peptides at pH 6 allowed separation of one major radioactive peak. The amino acid composition of this peak (molar ratio) was the following: aspartic acid, 2.01; threonine, 1.00; glutamic acid, 1.09; proline, 2.01; glycine, 1.11; alanine, 2.00; methionine, 0.22; isoleucine, 1.00; leucine, 2.01; phenylalanine, 1.00; lysine, 1.00; arginine, 0.33. A manual sequencing procedure unambiguously determined the structure of the peptide, which coincides with the peptide from Leu-82 to Lys-97 (LLDGFPRTIPQADAMK). The radioactivity was found exclusively in the seventh cycle of sequencing. Thus, the ^{14}C-labeled residue was identified as Arg-88. A minor radioactive peptide (less than 10% of the radioactivity of the major ^{14}C-labeled peptide) corresponding to the sequence RAAVK (residues 36–40) was also isolated.

Preparation and Characterization of RG88 Mutant Adenylate Kinase—Plasmid pEAK90, which contains the adenylate kinase gene on the high copy number plasmid pEMBL, directs the synthesis of high amounts of adenylate kinase protein. The same plasmid can be used for site-directed mutagenesis since it produces single-stranded DNA on superinfection with phage (16). We mutated arginine 88 to glycine using the method of Taylor *et al.* (27). Since the mutation generates two new restriction sites, one for *NciI* and the other for *KpnI* (recognition sequences 5′ CCGGG 3′ and 5′ GGTACC 3′, respectively), the mutant DNA was identified by restriction enzyme digests. Out of four clones, three were wild type, and one had a hybrid pattern (half-restricted by the two enzymes cited above). The DNA from this hybrid was purified by retransformation of competent cells of SMH50 and analyzed again by restriction analysis. Two out of six clones had the appropriate mutant restriction pattern. Furthermore, a lower adenylate kinase activity was found in crude extracts of those two mutants. The nucleotide sequence confirmed that a single mutation CGU (Arg-88) to GGU (Gly-88) was responsible for the observed phenotype. High amounts of mutant protein (RG88) were produced in *E. coli* strain SMH50, which also has a chromosomally encoded copy of the wild-type enzyme. Mutant and wild-type enzyme could easily be separated on a Blue-Sepharose column because RG88 adenylate kinase, in contrast to all wild-type adenylate kinases, does not bind to this matrix. This already indicated that RG88 protein might have a different affinity toward nucleotides because the Blue-Sepharose is believed to mimic the ligand-binding site on nucleotide-binding enzymes (32). The protein was purified to homogeneity by chromatofocusing and gel permeation chromatography (23, 28).

Amino acid analysis of the RG88 mutant adenylate kinase compared with that of wild-type protein corroborates the replacement of an arginine by a glycine residue as obtained by site-direct mutagenesis. To confirm the site of mutation, digests of both forms of adenylate kinase (wild-type and mutant strain) with TPCK-treated trypsin were analyzed by reverse-phase HPLC. Comparison of elution profiles derived from the mutant adenylate kinase (Fig. 3) with the wild-type protein (not shown) allows the identification of a particular peak in the mutant form. The amino acid analysis indicates that this peptide corresponds to a segment from Asn-79 to Lys-97, locating the site of the Arg→Gly substitution at position 88. All other peptides were identified by their amino acid composition; there was no evidence of the presence of another amino acid substitution in the molecule.

A comparison of the infrared spectra of the RG88 mutant with that of the wild-type adenylate kinase in the region of the conformation-sensitive protein amide I bands (33) indicated that there are no differences in the secondary structure of the two proteins that can be detected by this method.

Limited Proteolysis of Wild-type and RG88 Mutant Adenylate Kinase by Collagenase and Protection by ATP—Susceptibility of native proteins to inactivation by proteases is a sensitive probe of conformational changes induced by ligands or modifying reagents (34). *E. coli* adenylate kinase is specifically cleaved by collagenase from *A. iophagus*. The kinetics of inactivation obeys a first order reaction. At collagenase/adenylate kinase ratio of 1:50 (w/w), pH 7.4 and 30 °C, the rate constants were 1.9×10^{-3} s^{-1} and 2.2×10^{-3} s^{-1} for wild-type adenylate kinase and RG88 mutant, respectively. The electrophoretic patterns of collagenase-digested adenylate kinases were identical. ATP (as well as ADP or Ap_5A but not AMP) exerted significant protection against proteolysis (Fig. 4).

Thermal Stability of Wild-type and RG88 Mutant Adenylate Kinase—Fig. 5 shows typical differential scanning calorimetry traces for the wild-type and mutant adenylate kinase. The thermodynamic parameters obtained by evaluating calori-

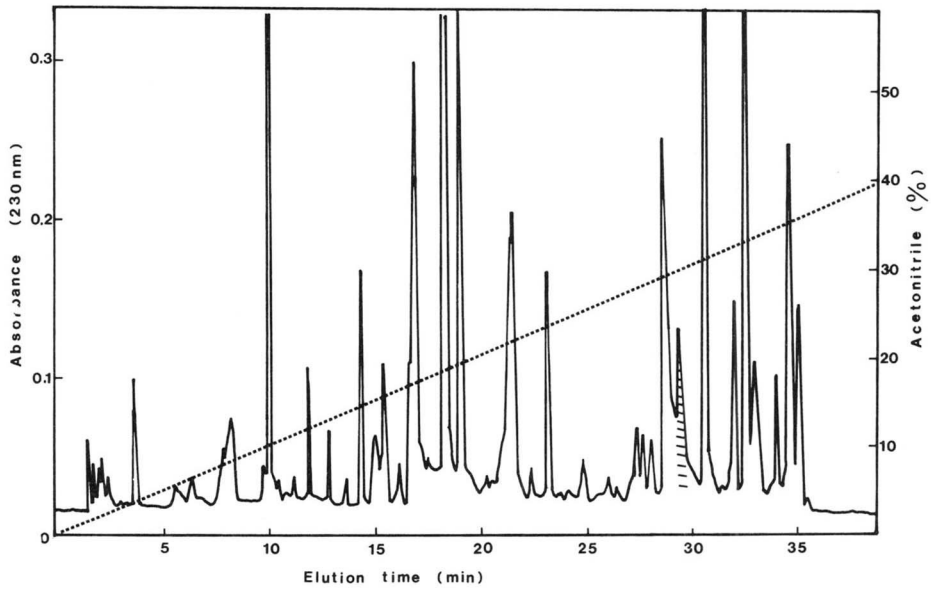

FIG. 3. **Separation of tryptic peptides from RG88 mutant adenylate kinase of *E. coli* by HPLC on a Nucleosil C-18 column.** Peptides were eluted with a linear gradient of 0–60% (v/v) acetonitrile (- - -) and detected by their absorbance at 230 nm (——). All peptides were analyzed for amino acid composition. The *dashed peak* indicates the peptide bearing the Arg→Gly substitution.

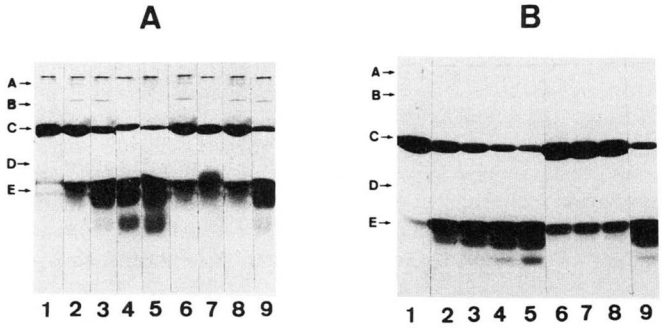

FIG. 4. **Proteolysis of wild-type (*A*) and RG88 mutant (*B*) adenylate kinase by collagenase and protection by nucleotides.** Adenylate kinase at 1 mg/ml in 50 mM Tris-HCl (pH 7.4) and 50 mM KCl was incubated at 30 °C with collagenase (20 μg/ml) in the absence (*lanes 1–5*) or presence of 4 mM ATP (*lane 6*), 4 mM ADP (*lane 7*), 1 mM Ap_5A (*lane 8*), and 4 mM AMP (*lane 9*). At different time intervals (5 s, *lane 1*; 5 min, *lane 2*; 10 min, *lane 3*; 15 min, *lane 4*; 20 min, *lanes 5–9*) 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 are the same as those indicated in the Fig. 1 legend.

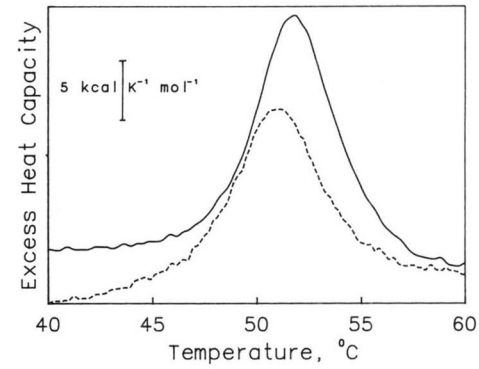

FIG. 5. **Differential scanning calorimetric traces of wild-type (*solid curve*) and RG88 mutant (*broken curve*) adenylate kinase.**

TABLE II

Thermodynamic parameters for the unfolding of wild-type and RG88 mutant adenylate kinase

Enzyme[a]	T_m	ΔH_{cal}	ΔH_{vH}	$\Delta H_{vH}/\Delta H_{cal}$	$\Delta G°$ [b]
	°C	*kcal/mol*			*kcal/mol*
Wild-type	51.8	95	158	1.7	0
RG88	51.0	75	161	2.1	0.2

[a] Protein concentration 1.4–1.6 mg/ml.

[b] Since repetitive experiments with the mutant protein 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.

metric data (35–37) are shown in Table II. The denaturation temperature (T_m) of the RG88 mutant is only 0.8 °C lower than that of the wild-type protein, whereas the calorimetric enthalpy of denaturation is 21% lower for the mutant enzyme as compared with that of wild-type protein. This can be attributed to small changes in noncovalent interactions which may result from the substitution of a glycine for a much larger cationic residue, arginine. For both enzymes, the ratio of van't Hoff and calorimetric enthalpies is above 1, suggesting intermolecular cooperation (37) presumably due to protein self-association. Muscle adenylate kinase in crystal form (10) as well as C1 peptide (the N-terminal segment of *E. coli* adenylate kinase) residues 1–76 (23) and the HG36 mutant of muscle adenylate kinase (38) in solution were shown to form "dimers" by intermolecular interactions. An additional parameter that can be derived from calorimetric data is the standard free energy ($\Delta G°$) of protein denaturation (35). The $\Delta G°$ values at 51.8 °C (the T_m of the wild-type adenylate kinase) between the two proteins differ by only 0.2 which indicates that the destabilization of protein structure caused by the replacement of Arg-88 by Gly is very small.

Dissociation and Kinetic Constants of the Adenylate Kinase RG88 Mutant—Preliminary assays under standard conditions (1 mM ADP or 1 mM ATP + 0.3 mM AMP) of pure RG88 mutant adenylate kinase of *E. coli* indicated that its activity is only 0.1% of that of wild-type protein in the sense of ADP or ATP formation.

Table III shows the kinetic parameters of wild-type and RG88 mutant adenylate kinase. The apparent K_m value for ATP is increased by a factor of 5 in RG88 adenylate kinase. The drastic increase of the apparent K_m for AMP (almost 100-fold) in the Arg-88→Gly substitution was rather unexpected. It is possible that the tight coupling of the two nucleotide-binding sites in adenylate kinase is responsible for

TABLE III

Kinetic parameters of wild-type and RG88 mutant adenylate kinase of E. coli

The reaction medium contained either 50 mM Tris-HCl (pH 7.4), 100 mM KCl, 1 mM glucose, 0.4 mM $NADP^+$, 2 mM $MgCl_2$, different concentrations of ADP, and 5 units each of hexokinase and glucose-6-phosphate dehydrogenase or 100 mM Tris-HCl (pH 7.4), 0.2 mM NADH, 0.4 mM phosphoenolpyruvate, 80 mM KCl, 2 mM $MgCl_2$, different concentrations of ATP and AMP, and 10 units of each lactate dehydrogenase and pyruvate kinase. The reaction was started with adenylate kinase. K_m^{ADP} and V_m^{ADP} were determined from plots of $1/v$ *versus* $1/ADP^2$, which assumes that the two molecules of ADP bind to the enzyme with the same affinity. The apparent K_m for ATP and for AMP was determined at a single fixed concentration of cosubstrate (0.3 mM AMP and 1 mM ATP, respectively). The $V_m^{ATP,AMP}$ was obtained by extrapolating the reaction rates for infinite concentrations of ATP and AMP and assuming that the concentration of one nucleotide substrate does not affect the apparent K_m 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*	*μM*	*μmol/min/mg of protein*
Wild-type	92	433	71	26	830
RG88 mutant	232	0.52	350	2200	9.4

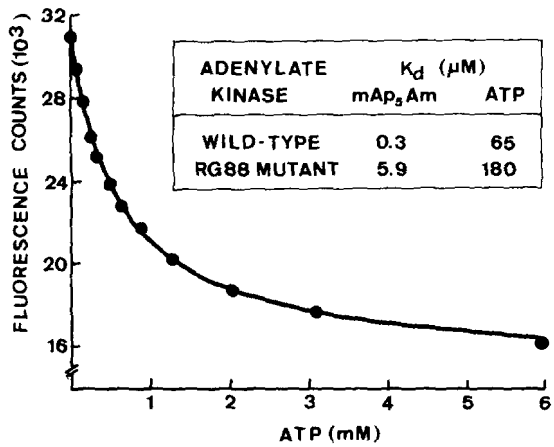

FIG. 6. **Displacement by ATP of the bound mAp_5Am from RG88 mutant adenylate kinase.** A 15 μM solution of mutant protein with 1.12 μM mAp_5Am in the presence of 10 mM Mg^{2+} was titrated with increasing concentrations of ATP. The displacement of bound analogue from the protein by ATP was measured as a decrease in fluorescence emission at 440 nM (excitation wavelength 360 nm) and analyzed as described under "Experimental Procedures." *Inset* shows the calculated K_d for the Mg^{2+}-complexed ATP and mAp_5Am of wild-type and RG88 mutant adenylate kinase.

this fact. Tomasselli and Noda (39) observed that the K_m for AMP of yeast adenylate kinase was 17 times higher in the presence of MgGTP than in the presence of MgATP. For reasons that are unclear at this moment, the maximum rate of catalysis is reduced 100-fold in the sense of ADP formation and 800-fold in the sense of ATP formation following Arg-88→Gly substitution.

We also determined the affinity of the wild-type and RG88 mutant adenylate kinase for mAp_5Am and ATP (Fig. 6). The K_d for fluorophore was determined directly by titrating enzyme to a fixed amount of mAp_5Am. The fluorophore was then displaced from the enzyme with ATP, and the known K_d for mAp_5Am was used to calculate the K_d of the adenylate kinase/ATP complex (Fig. 6, *inset*). Equilibrium dialysis confirmed the fluorescence measurements (a K_d of 200 μM for [α-^{32}P]ATP binding to RG88 mutant was found). The affinity of AMP for the mutant protein was too low to be measured accurately by equilibrium dialysis and could also not be measured by fluorescence.

DISCUSSION

X-ray structure investigation of mammalian cytosolic adenylate kinase (AK1) crystallized in the absence of nucleotides indicated a bilobed structure where the two lobes are separated by a big cleft (6). Adenylate kinase, like other phosphotransferases, undergoes a substrate-induced conformational change (40). For hexokinase (41) and the yeast adenylate kinase/Ap_5A complex (9), this conformational change results in the closing of the cleft. In AK1, several arginine residues (Arg-44, Arg-97, Arg-128, Arg-132, Arg-138, and Arg-149) line the cleft. According to the homology plot of Schulz *et al.* (5), all of these arginines are situated in regions that are highly conserved in the primary structure and correspond to Arg-36, Arg-88, Arg-119, Arg-123, Arg-131, and Arg-167 in adenylate kinase from *E. coli*. Arg-36, Arg-119, Arg-123, and Arg-131 (using the *E. coli* numbering and assuming that the catalytic residues are identical in the tertiary structure, see Ref. 9) are situated at the entrance of the cleft, and Arg-88 and Arg-167 are located inside.

All of these arginines are likely to participate in binding of the negatively charged phosphate group and perhaps catalysis. Arg-88 belongs to a sequence which forms a loose turn around Pro-87 and faces the glycine-rich loop at the N terminus of the protein and which itself appears implicated in the nucleotides binding (5). The spatial proximity of these two flexible sequences makes very likely a concerted action of their residue side chains in binding/adjustment of the nucleotides or phosphate transfer. Arg-88 can be modified specifically with phenylglyoxal. Protection by ATP, ADP, and Ap_5A seems to indicate that this arginine residue is either involved in the binding of ATP or is situated close to the binding site of ATP such that the presence of the substrate can block access to this residue. However, AMP was shown to exert also a slight protecting effect against inactivation by phenylglyoxal. This effect was enhanced by Mg^{2+} ions, in agreement with earlier data. Berghaüser (19) had found that modification of AK1 with phenylglyoxal at 37 °C can be inhibited by AMP in the presence of Mg^{2+}. That the modified enzyme is inactivated seems to indicate that Arg-88 is also involved in catalysis or that the binding of substrate is sterically hindered after modification.

To clarify the role of Arg-88, we have mutated it to glycine. The flexibility of glycine allows it to adopt a backbone conformation accommodating easily a β-turn too. Therefore Arg→Gly substitution was expected to conserve the native type structure in the mutant protein. That the protein is expressed in amounts similar to wild-type enzyme indicates already that the mutant protein is able to form a stable structure because it is known that incorrectly folded proteins are quickly degraded in *E. coli* (42). Furthermore, our results with differential scanning calorimetry, Fourier transform infrared spectroscopy, and susceptibility to proteolysis show that the protein has a secondary/tertiary structure that is close to that of wild-type protein. The activity of the enzyme is, however, reduced by 2 and 3 orders of magnitude in the sense of ADP and ATP formation, respectively, which clearly shows that Arg-88 is indeed critical for catalysis.

It is interesting to recall that substitution of the immediately preceding proline residue (Pro-87) with serine conserved the catalytic activity of the wild-type enzyme to a much higher extent. However, the latter mutation altered the conformation of adenylate kinase considerably, leading to an increased sensitivity to thermal denaturation or proteolytic degradation

(43–45). This was explained by the specific conformation role played by *cis*-proline in this segment of the protein (10). Whereas the dissociation constant of ATP and the K_m of ATP are only moderately increased (3–5-fold), in the RG88 mutant adenylate kinase the K_m value for AMP is more drastically increased, almost 100-fold. This could indicate that Arg-88 is involved in binding of AMP or that the two nucleotides-binding sites in adenylate kinase are tightly coupled. It is difficult at this step to favor one of these two possibilities since other variants of bacterial or mammalian adenylate kinase obtained by site-directed mutagenesis had altered kinetic constants for both nucleotide substrates (16, 38).

From a determination of the kinetic and binding constants, one can thus conclude that it is not the low affinity of substrates per se that lead to a decrease in catalytic activity. We have shown earlier that mutants in the nucleotide-binding loop of adenylate kinase which drastically increased K_m values of both substrates are nevertheless very active enzymes (16). Thus, we have to conclude that Arg-88, which is situated in the most highly conserved region of the protein, plays a critical, albeit not essential role in catalysis of adenylate kinase, either in the binding of a particular substrate by inducing the conformational change necessary for the induced fit or by stabilizing the transferable γ-phosphate of ATP in the transition state.

Acknowledgments—We thank K. C. Holmes and A. Ullmann for continuous support, S. Fermandjian for helpful comments on the manuscript, R. S. Goody for the fluorescent mAp_5Am analogue, P. Lang for the preparation of the oligonucleotide, B. T. Nhung for expert technical assistance, and L. Girardot for excellent secretarial help.

REFERENCES

1. Banks, R. D., Blake, C. C. F., Evans, P. R., Haser, R., Rice, D. W., Hardy, G. W., Merrett, M., and Phillips, A. W. (1979) *Nature* **279,** 773–777
2. Evans, P. R., and Hudson, P. J. (1979) *Nature* **279,** 500–504
3. Bhat, T. N., Blow, D. M., Brick, P., and Nyborg, J. (1982) *J. Mol. Biol.* **158,** 699–709
4. Walker, J. E., Saraste, M., Runswick, M. J., and Gay, N. J. (1982) *EMBO J.* **1,** 945–951
5. Schulz, G. E., Schiltz, E., Tomasselli, A. G., Frank, R., Brune, M., Wittinghofer, A., and Schirmer, R. H. (1986) *Eur. J. Biochem.* **161,** 127–132
6. Schulz, G. E., Elzinga, M., Marx, F., and Schirmer, R. H. (1974) *Nature* **250,** 120–123
7. Sachsenheimer, W., and Schulz, G. E. (1977) *J. Mol. Biol.* **114,** 23–36
8. Pai, E. F., Sachsenheimer, W., Schirmer, R. H., and Schulz, G. E. (1977) *J. Mol. Biol.* **114,** 37–45
9. Egner, U., Tomasselli, A. G., and Schulz, G. E. (1987) *J. Mol. Biol.* **195,** 649–658
10. Dreusicke, D., Karplus, P. A., and Schulz, G. E. (1988) *J. Mol. Biol.* **199,** 359–371
11. Fry, D. C., Kuby, S. A., and Mildvan, A. S. (1985) *Biochemistry* **24,** 4680–4694
12. Fry, D. C., Kuby, S. A., and Mildvan, A. S. (1987) *Biochemistry* **26,** 1645–1655
13. Fry, D. C., Byler, D. M., Susi, H., Brown, E. M., Kuby, S. A., and Mildvan, A. S. (1988) *Biochemistry* **27,** 3588–3598
14. Hamada, M., Palmieri, R. H., Russell, G. A., and Kuby, S. A. (1979) *Arch. Biochem. Biophys.* **195,** 155–177
15. Tagaya, M., Yagami, T., and Fukui, T. (1987) *J. Biol. Chem.* **262,** 8257–8261
16. Reinstein, J., Brune, M., and Wittinghofer, A. (1988) *Biochemistry* **27,** 4712–4720
17. Borders, C. L., Jr., and Riordan, J. F. (1975) *Biochemistry* **14,** 4699–4704
18. Powers, S. G., and Riordan, J. F. (1975) *Proc. Natl. Acad. Sci. U. S. A.* **72,** 2616–2620
19. Berghäuser, J. (1975) *Biochim. Biophys. Acta* **397,** 370–376
20. Berghäuser, J., and Schirmer, R. H. (1978) *Biochim. Biophys. Acta* **537,** 428–435
21. Viale, A. M., and Vallejos, R. H. (1985) *J. Biol. Chem.* **260,** 4958–4962
22. Lecroisey, A., Keil-Dlouha, V., Woods, R. R., Perrin, D., and Keil, B. (1975) *FEBS Lett.* **59,** 167–172
23. Saint Girons, I., Gilles, A. M., Margarita, D., Michelson, S., Monnot, M., Fermandjian, S., Danchin, A., and Bârzu, O. (1987) *J. Biol. Chem.* **262,** 622–629
24. LeClerk, I. E., Saran, B. R., and Allen, R., Jr. (1984) *J. Mol. Biol.* **180,** 217–227
25. Brune, M., Schumann, R., and Wittinghofer, F. (1985) *Nucleic Acids Res.* **13,** 7139–7151
26. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
27. Taylor, J. W., Ott, J., and Eckstein, F. (1985) *Nucleic Acids Res.* **13,** 8765–8785
28. Bârzu, O., and Michelson, S. (1983) *FEBS Lett.* **153,** 280–284
29. Yang, C. Y., Panly, K., Kratzin, H., and Hilschmann, N. (1981) *Hoppe-Seyler's Z. Physiol. Chem.* **362,** 1131–1146
30. Chang, J. Y., Brauer, D., and Whittman-Liebold, B. (1978) *FEBS Lett.* **93,** 205–214
31. Yang, P. W., Mantsch, H. H., Arrondo, J. L. R., Saint Girons, I., Guillou, Y., Cohen, G. N., and Bârzu, O. (1987) *Biochemistry* **26,** 2706–2711
32. Thomson, S. T., Cass, K. H., and Stellwagen, E. (1975) *Proc. Natl. Acad. Sci. U. S. A.* **72,** 669–672
33. Surewicz, W. K., and Mantsch, H. H. (1988) *Biochim. Biophys. Acta* **952,** 115–130
34. Keil, B. (1982) in *Methods in Sequence Analysis* (Elzinga, M., ed) pp. 291–304, Humana Press, Inc., Clifton, NJ
35. Privalov, P. L., and Khechinashvili, N. N. (1974) *J. Mol. Biol.* **86,** 665–684
36. Velicelebi, G., and Sturtevant, J. M. (1979) *Biochemistry* **18,** 1180–1186
37. Sturtevant, J. M. (1987) *Ann. Res. Phys. Chem.* **38,** 463–488
38. Tian, G., Sanders, C. R., II, Kishi, F., Nakazawa, A., and Tsai, M. D. (1988) *Biochemistry* **27,** 5544–5552
39. Tomasselli, A. G., and Noda, L. (1983) *Eur. J. Biochem.* **132,** 109–115
40. Anderson, C. M., Zucker, F. H., and Steitz, T. A. (1979) *Science* **204,** 375–380
41. Shoham, M., and Steitz, T. A. (1982) *Biochim. Biophys. Acta* **705,** 380–384
42. Goff, S. A., and Goldberg, A. L. (1985) *Cell* **41,** 587–595
43. Guiso, N., Michelson, S., and Bârzu, O. (1984) *J. Biol. Chem.* **259,** 8713–8717
44. Gilles, A. M., Saint Girons, I., Monnot, M., Fermandjian, S., Michelson, S., and Bârzu, O. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 5798–5802
45. Haase, G. H. W., Brune, M., Reinstein, J., Pai, E., Pingoud, A., and Wittinghofer, A. (1989) *J. Mol. Biol.*, in press