Mutations in the Nucleotide Binding Loop of Adenylate Kinase of *Escherichia coli*

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ABSTRACT: The *adk* gene of *Escherichia coli* has been used to overexpress the adenylate kinase protein in two ways: (1) by cloning the *adk* gene with its own promoter into pEMBL plasmids, which have an increased copy number, and (2) by deleting the adk promoter and cloning the gene behind the regulatable tac promoter. Adenylate kinase comprises up to 40% of the soluble cellular extracts from *E. coli* strains containing these plasmids. Mutations have been introduced into the gene by site-directed mutagenesis to exchange amino acids in the nucleotide binding loop, which is highly conserved in many mononucleotide binding proteins. The mutation of $Lys_{13} \rightarrow Gln$ is nearly inactive, whereas the $Pro_9 \rightarrow Leu$ and the $Gly_{10} \rightarrow Val$ mutant proteins have an increased K_m for both substrates and a V_{max} that is similar to wild type. Proton NMR measurements of the proteins show that a major structural change seems to have taken place for the $Pro_9 \rightarrow Leu$ and $Gly_{10} \rightarrow Val$ mutants. The results are discussed in the light of the kinetic mechanism for adenylate kinase and the three-dimensional structure of the protein.

Loops and turns are more and more being recognized as important secondary structure elements of proteins [for reviews, see Rose et al. (1985), Richardson (1981), and Kabsch and Sander (1983)]. Loops are structurally not well defined and may be regarded as turns with more than four residues, with no or only one internal hydrogen bond. These structural elements, also termed bends, are regions of high curvature of the protein backbone. Very often they are hydrophilic, situated at the surface of the protein, and seem to be important sites of immunogenic recognition and location of posttranslational modification.

Many adenine and guanine nucleotide binding proteins, and possibly the proteins that bind other nucleotides, share a common loop, which connects a β-sheet strand with an α-helix and has been termed the nucleotide binding loop (Gay & Walker, 1983; Wierenga & Hol, 1983; Halliday, 1983; Leberman & Egner, 1984; Möller & Amons, 1985; Hurley et al., 1984; Fry et al., 1986). The common sequence for this loop is Gly-Xaa-Yaa-Yaa-Xaa-Gly-Lys-Zaa, where Xaa can be any amino acid, Yaa very often is a glycine or proline, and Zaa is very often serine for GTP/GDP binding proteins and threonine (sometimes behind another glycine) for many ATP/ADP binding enzymes. Mutation of the Yaa residue 12 from glycine to any other amino acid except proline in the *ras*-gene-encoded proteins termed p21 has been shown to be responsible for the oncogenic activation of this group of proteins (Tabin et al., 1982; Reddy et al., 1982; Dhar et al., 1982; Tsuchida et al., 1982; Seeburg et al., 1984). Substitution of the neighboring amino acid Gly_{13} also has transforming consequences (Fasano et al., 1984; Liu et al., 1987).

Adenylate kinase has been found to be necessary for growth in *Escherichia coli*, and it is generally assumed that it is essential for all living organisms (Lipmann, 1981). The nucleotide binding loop in the adenylate kinase family is very highly conserved in primary structure and has the sequence Gly-Xaa-Pro-Gly-Xaa-Gly-Lys-Gly-Thr with an unusually high glycine content (Heil et al., 1974; Schulz et al., 1986). The three-dimensional structure of yeast and porcine adenylate kinase has been determined, see Figure 1A (Schulz et al., 1974; Pai et al., 1977; Egner et al., 1987), and the structure of the *E. coli* enzyme is also being studied (Schulz, unpublished results). As shown in Figure 1B, the glycine-rich loop is seen in the refined structure of the pig cytosolic AK1[1] (Dreusicke et al., 1988) as a giant anion hole for a sulfate ion, which is believed to mimic a phosphate ion (Dreusicke & Schulz, 1986). In the yeast adenylate kinase–Ap_5A inhibitor complex the β,γ-phosphate residues are seen in contact with this loop (Egner et al., 1986). Here we have mutated various key residues in the nucleotide binding loop of adenylate kinase from *E. coli* analogous to the oncogenic mutations in p21. These mutations in adenylate kinase are highly active, although they seem to produce major structural changes, as is apparent in the aromatic portion of the NMR spectrum of the mutants. To be able to purify possible mutant proteins with low activity against a background of chromosomally encoded adenylate kinase, we devised an expression system whereby the plasmid-encoded enzyme comprises up to 40% of the soluble extract.

Materials and Methods

Strains and Plasmids. The temperature-sensitive *E. coli* strain CV2 was obtained from B. Bachman (Genetic Stock Center, Yale University). Strain CK600 (*supE*, *hsdM*$^+$, *hsdR*$^-$) was kindly provided by H. Hoffmann-Berling. The *lacI*^{Q}-containing strain RR1ΔM15 has been described earlier (Tucker et al., 1986). Plasmid pEMBL9$^+$ (Dente et al., 1983) was provided by G. Cesareni. The tac promoter vector pJF118u, which has later been called pJF118EH (Fürste et al., 1986), was obtained from E. Lanka, Berlin. pfdA8 (Geider et al., 1985) was from K. Geider, and pHC624 (Boros et al., 1984) was obtained from I. Boros. Plasmid pAK601 has been described earlier (Brune et al., 1985). M13mp9rev, BMH71-mutS, and MK30-3 (Kramer et al., 1984) were kindly

[1] Abbreviations: AK, adenylate kinase (EC 2.7.4.3); AKe, adenylate kinase from *Escherichia coli*; AK1, mammalian cytosolic enzyme; *adk*, adenylate kinase gene of *E. coli*; Ake(wt), wild-type *E. coli* enzyme; NMR, nuclear magnetic resonance; TEA, triethylammonium acetate; DMT, dimethoxytrityl; IPTG, isopropyl β-D-thiogalactoside; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; PEP, phosphoenolpyruvate; BSA, bovine serum albumin; HPLC, high-performance liquid chromatography; DSS, sodium 4,4-dimethyl-4-silopentane-1-sulfonate; 3-D, three dimensional; 1-D, one dimensional; bp, base pair. Mutant adenylate kinases from *E. coli* for PL9 with a $proline_9 \rightarrow$ leucine mutation, GV10 with $glycine_{10} \rightarrow$ valine, and KQ13 with $lysine_{13} \rightarrow$ glutamine.

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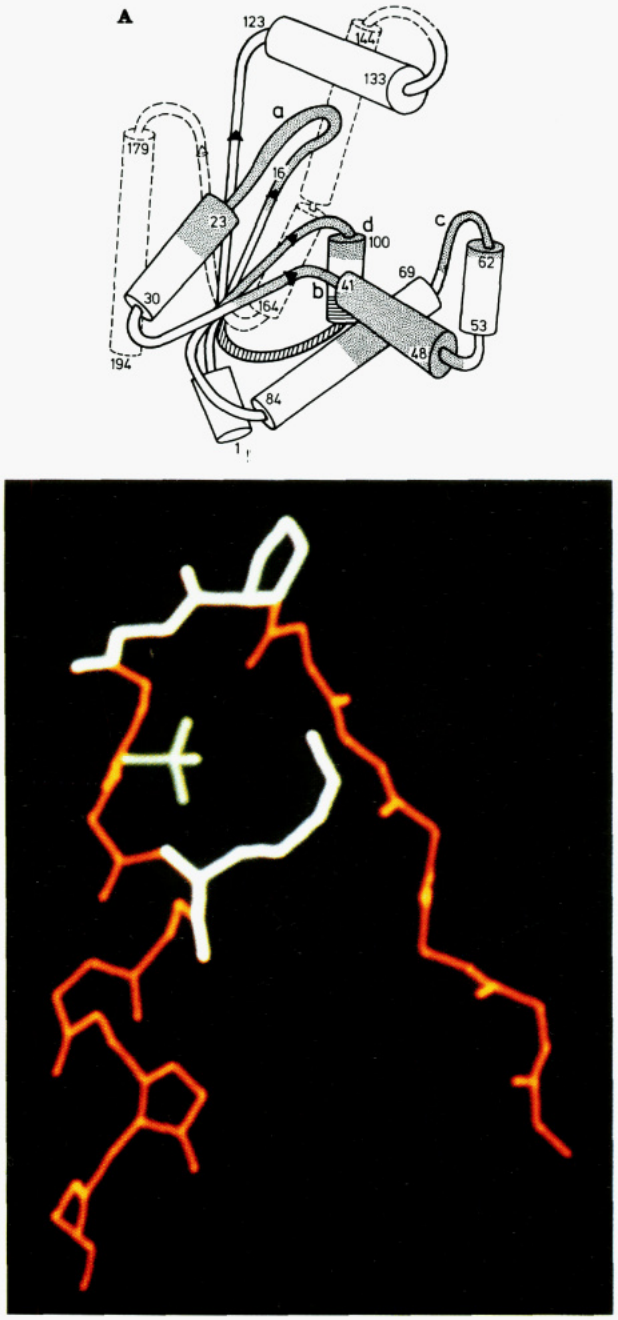

FIGURE 1: Structure of adenylate kinase. (A) A sketch of the pig muscle AK1 structure using cylinders for helices and arrows for β-sheets taken from Frank et al. (1984). Shaded areas indicate the regions of high homology between all AK's, which are believed to constitute the substrate binding sites. (B, bottom) A computer graphics representation of the polypeptide backbone of residues 4–19 of AKe (corresponding to residues 12–27 of AK1), whose primary structure is shown in Table I. The amino acids mutated in this study (in white) are shown with their side chains together with a sulfate ion (in green), which has been found in the crystal structure of AK1 (Dreusicke & Schulz, 1986). The refined X-ray coordinates were given to us by G. Schulz.

provided by W. Kramer. SMH50 (Taylor et al., 1985) was obtained from F. Eckstein.

Cloning Methods. Restriction enzymes and T4 DNA ligase were obtained from Boehringer Mannheim. DNA polymerase I large fragment (Klenow fragment) was from Serva, Heidelberg. Enzyme buffers were used according to Maniatis et al. (1982). Transformation procedures were carried out as described by Hanahan (1983) and Maniatis et al. (1982), respectively.

Synthesis and Purification of Oligonucleotides. Oligonucleotides were synthesized according the phosphoamidite method with a commercial DNA synthesizer (Cyclone Biosearch). Products were purified in a two-step procedure with HPLC (Beckman) using a C_{18}-ODS column and the following parameters: flow rate, 2 mL/min; elution gradient, 100 mM triethylammonium acetate (TEA) in 20–40% acetonitrile for 20 min for the DMT-oligonucleotide and in 5–20% acetonitrile for 30 min for the detritylated oligonucleotide.

Site-Directed Mutagenesis. Mutagenesis procedures were carried out with M13mp9 containing the *adk* gene according to the gapped-duplex method (Kramer et al., 1984) essentially as described. For pEAK90 single-stranded DNA the method of Taylor et al. (1985) was used. The mutant clones were identified by restriction enzyme digests, where possible, or by sequencing with the chain termination method (Sanger et al., 1977). A fragment of the mutated *adk* gene containing the point mutation was cloned into pEAK90, where the corresponding wild-type fragment had been removed. Sequences of the resulting mutated pEAK plasmids between the restriction sites used for cloning were verified by the chemical modification method (Maxam & Gilbert, 1980) using a solid support as described (Rosenthal et al., 1986).

Cell Growth and Cell-Free Extract. Cells were grown in standard I medium (Merck, Darmstadt) containing up to 100 μg/mL carbenicillin. In the case of those clones containing plasmids with the tac promoter, between 50 and 500 μM IPTG (Serva, Heidelberg) was added (see Results). Cell-free (crude) extract for enzyme activity measurements and SDS gels was prepared as described before (Brune et al., 1985). Adenylate kinase activity was determined by optical tests either for the forward reaction, i.e., the ADP-forming direction (Berghäuser, 1975), or for the reverse reaction as described (Brune et al., 1985). Protein concentration in crude extract was measured by the UV absorption method of Ehresmann et al. (1973).

Protein Purification. After lysis of cells as described by Leberman et al. (1980), the soluble extract was loaded onto a Blue Sepharose affinity column (Pharmacia) equilibrated with 50 mM Tris-HCl, pH 7.5, and subsequently eluted of proteins with 0–1 M KCl/50 mM Tris-HCl, pH 7.5. Fractions with sufficient adenylate kinase activity as judged by the coupled assay of Berghäuser (1975) were pooled and dialyzed against 50 mM Tris-HCl, pH 7.5. If necessary, this pool was further purified on a DEAE ion-exchange column (Merck) and eluted with 0–0.5 M KCl in the same buffer system as described for Blue Sepharose. The advantage of using a salt gradient in the first purification step in contrast to the purification scheme first described by Barzu and Michelson (1983) is that it yields nucleotide-free protein (as judged from HPLC analysis).

Steady-State Kinetics. The solutions for the coupled colorimetric assay of the forward reaction (Berghäuser, 1975) were as follows: 2 mM $MgCl_2$, 100 mM Tris-HCl, pH 7.5, 200 μM NADH, 400 μM PEP, and 80 mM KCl. For the determination of V_{max} and K_m for AMP and ATP, the ATP and AMP concentrations were held constant at 1 mM and 300 μM, respectively. The final volume in the test cuvette was 1 mL temperated to 25 °C, containing 10 units from each of the helper enzymes lactate dehydrogenase and pyruvate kinase. The amount of adenylate kinase added to start the reaction was between 50 and 100 ng per reaction. Diluted enzyme solutions with 5–10 μg/mL were stabilized with 0.2 mg of BSA/mL. Helper enzymes and BSA alone produced negligible activity. Enzyme concentrations of adenylate kinase stock solutions were determined according to Bradford (1976) using

BSA as standard. The decrease of absorption at $\lambda = 340$ nm due to the consumption of NADH in the coupled assay was recorded on a UV-260 Shimadzu spectrophotometer, and the resulting slope at $t = 15$ s was taken to determine the initial velocity. One unit of enzyme activity is defined as the consumption of 1 μmol of AMP/min.

Analysis of Nucleotides. ATP, AMP, GMP, and IMP (from Aldrich) were of the highest purity available. The purity of ATP and AMP was analyzed by HPLC on an hydrophobic C_{18} column (Bishop) under the following conditions: flow rate, 2 mL/min; elution buffer, 50 mM potassium phosphate, pH 6.5, 3% CH_3CN, and 0.2 mM $[N(C_4H_9)_4]Br$. Purity of ATP and AMP were 99% and 99.9%, respectively. The same method was used to measure the amount of nucleotides in the protein solutions used for NMR measurements.

Preparation of NMR Probes. A total of 15 mg of protein was dialyzed for 5 h at 4 °C against 2 L of 40 mM potassium phosphate, pH 7.5, to which a spoonful of mixed-bed ion-exchanger Amberlite ICR-718 (Serva) was added. The resulting protein solution was lyophilized, dissolved in about 2–3 mL of D_2O (Merck) to exchange dissociatable protons, and after 6 h at 4 °C again lyophilized. The freeze-dried enzyme was dissolved in 0.5 mL of D_2O (Sigma) just before measurement, resulting in an enzyme concentration of about 0.7 mM.

NMR Measurements. Samples were measured on an 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

Cloning and Overexpression. We have described earlier the cloning of the *E. coli* adenylate kinase gene (Brune et al., 1985). For the mutation of the *adk* gene we wanted to get maximum expression of the gene product, because otherwise the small intrinsic activity of the chromosomally encoded adenylate kinase would interfere with the results on partially active mutant proteins. For this we pursued two different strategies: We tried to introduce the gene both into plasmids bearing a strong and inducible promoter and into plasmids with a high copy number, taking advantage of the *adk* gene's own promoter. Although the location of the promoter for the adenylate kinase gene has not been determined experimentally, we have tentatively assigned a TTGAGG and a GTTTAT sequence 76 and 49 bp upstream of the ATG translation codon site as the possible −35 and −10 promoter sequences, respectively.

We cloned the *adk* gene as a 1.1-kb *Pvu*II-*Sal*I fragment from the plasmid pAK601 into the vector pJF118u (Fürste et al., 1986), which contains the strong regulatable tac promoter in front of a multiple cloning site, as shown in Figure 2. The plasmid also encodes the mutated *lac* repressor gene ($lacI^Q$) to ensure tight control of expression. The resulting clone, pFAK1, was tested for expression in a crude extract. Figure 3 shows that the expression of the protein from plasmid pFAK1 is rather high in the uninduced state (comparable to pAK601). Surprisingly, it drops sharply after the induction with IPTG. We tested different concentrations of IPTG, and in all cases we observed decreased expression of adenylate kinase; this could be detected both by activity measurements and by SDS gel electrophoresis in crude cell extracts (Figure 3). Constructions containing the *adk* gene behind the P_L promoter of phage λ also showed no increase of adenylate kinase expression after induction and enzyme activity was again high in the uninduced state (data not shown).

From these results we concluded that the adk promoter, which we believe is encoded within the *Pvu*II/*Sal*I fragment of pAK601 (Brune et al., 1985) and has here been cloned behind the tac promoter in pFAK1, might be responsible for AKe production in the uninduced state. After induction with IPTG, transcription from the tac promoter might interfere with transcription from the adk promoter, leading to reduced expression of the adenylate kinase. To test this hypothesis, we changed the cryptic *Cla*I site (ATCGCT), which is located between the adk promoter region and the structural gene, to ATCGAT by site-directed mutagenesis. We could now separate the presumed adk promoter from the structural gene. We then reintroduced the *adk* gene as a 920-bp *Cla*I/*Sal*I fragment into pJF118u (Figure 2). With the new construction pJFAK11 we tested again for expression of the adenylate kinase gene. As can be seen in Figure 3, very little protein was then expressed in the uninduced state and much more protein in the induced state. Figure 3 also shows that this plasmid produces more adenylate kinase than pAK601 (about 3–4-fold), which contains the *adk* gene with its own promoter.

In a second approach we cloned the *adk* gene into different plasmids with a high copy number, like pdfA8 (Geider et al., 1985), pHC624 (Boros et al., 1984), and the vectors of the pEMBL family (Dente et al., 1983). We were able to clone the *adk* gene only into the vectors pEMBL8 and -9. In pEAK90 the *Pvu*II/*Sal*I fragment from pAK601 containing the *adk* gene and its promoter has been inserted into the multiple cloning site of pEMBL9, as outlined in Figure 2. A derivative of this plasmid is pEAK91 in which a *Cla*I site has been introduced by site-directed mutagenesis (see above and Figure 2). Both constructions were tested for expression of protein. We observed that cells containing pEAK90 (and pEAK91) showed a drastic increase in expression of adenylate kinase as compared to pAK601 cells (4–5-fold amount of protein and enzyme activity). The amount of the enzyme comprises up to 40% of the soluble extract (Figure 2). This high expression of adenylate kinase is independent of the orientation of the lac operator of pEMBL9, because it could also be observed by cloning the gene into pEMBL8 using the same restriction sites, where the *adk* gene is inverted with respect to the *lacZ* gene (data not shown). This suggests that the high expression of the *adk* gene is related to the copy number of the gene. The copy number of pUC and pEMBL plasmids has been determined to be 128 at 37 °C (Miki et al., 1987), whereas the copy number of pBR322, from which pAK601 is derived, is approximately 25. The copy number is higher because the gene encoding rop, the repressor of the primer of replication, has been deleted during the construction of these vectors (Twigg & Sheratt, 1980; Cesareni et al., 1982; Yanisch-Perron et al., 1985). Thus the increase in adenylate kinase expression can be quantitatively correlated with the increased copy number of the *adk* gene in these cells, as evidenced by the AKe activity in crude cell extracts (Figure 3). This seems to indicate that the *adk* gene has a constitutive promoter and that there is no posttranslational control of the concentration of AKe in the bacterial cell.

Mutation of the adk Gene and Isolation of Mutant Proteins. Table I lists the oligonucleotides that were used for the mutagenesis of the *adk* gene along with the sequence of the gene and derived amino acid sequence of the nucleotide binding region. The same region of the protein is shown in Figure 1B as the tracing of the polypeptide chain (in red), together with the complete α-carbons of $proline_9$, $glycine_{10}$, and $lysine_{13}$ (in

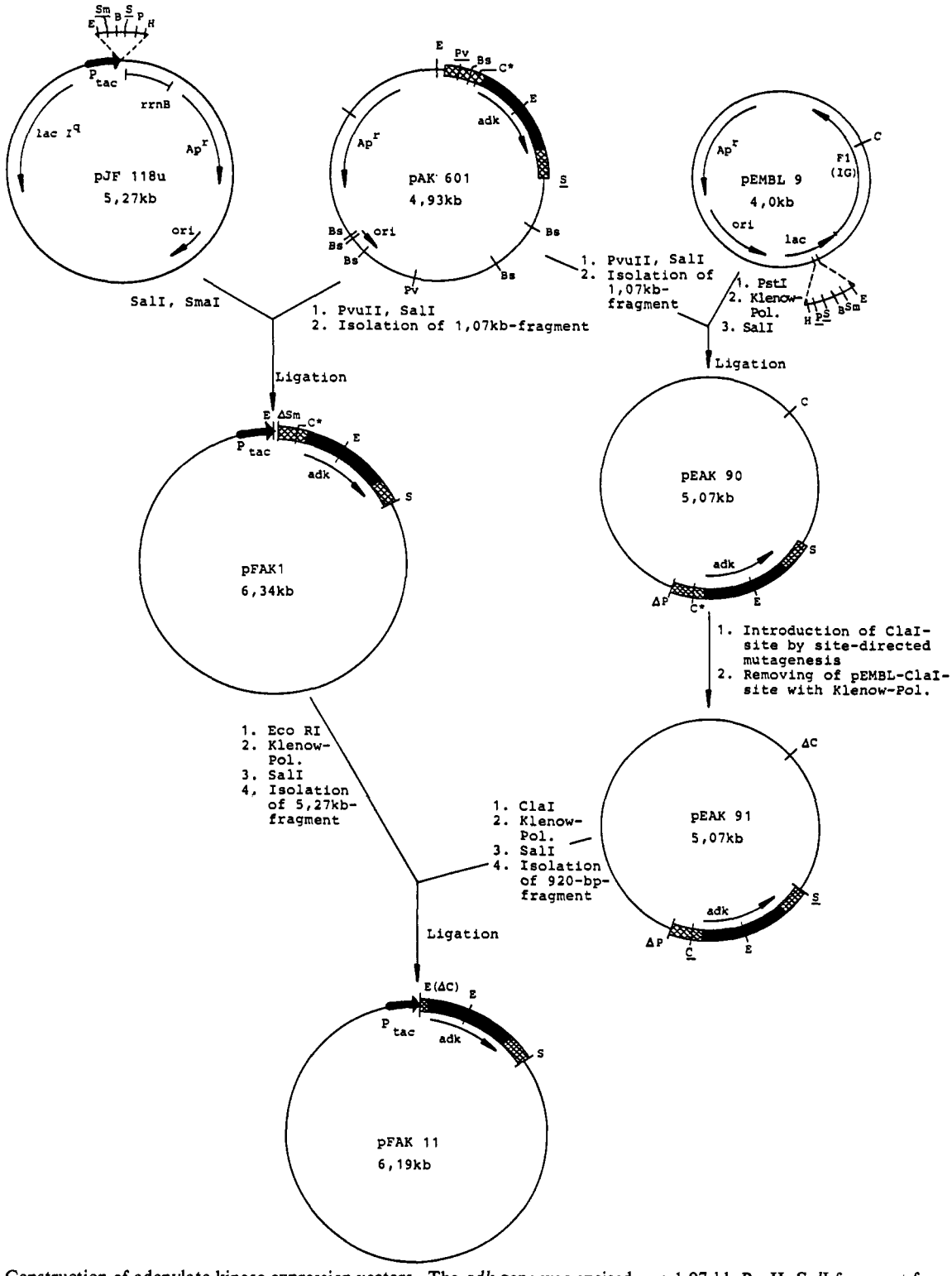

FIGURE 2: Construction of adenylate kinase expression vectors. The *adk* gene was excised as a 1.07-kb *Pvu*II–*Sal*I fragment from pAK601, the construction of which has been described earlier (Brune et al., 1985). This fragment was ligated into the P_{tac}-containing plasmid pJF118u (Fürste et al., 1986), cut with *Sal*I and *Sma*I, and into pEMBL9$^+$ (Dente et al., 1983), cut with *Pst*I and *Sal*I. The new constructions pFAK1 and pEAK90 were verified by restriction digestions and by expression analysis with adenylate kinase activity measurements (see Figure 3). A *Cla*I site was introduced by site-directed mutagenesis into pEAK90. The *Cla*I site in the F1(IG) part of the vector was removed by digesting with *Cla*I and filling up with Klenow polymerase to produce pEAK91. This was cut with *Cla*I and *Sal*I, and the 920-bp fragment containing the *adk* gene without promoter was ligated to the 5.27-kb *Eco*RI-*Sal*I fragment of pFAK1 to generate pFAK11. Abbreviations for restriction enzymes: B, *Bam*HI; Bs, *Bst*NI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I; Sm, *Sma*I. Restriction enzyme sites in the plasmids used for the constructions are underlined.

green). Although the gapped-duplex method (Kramer et al., 1984) for site-directed mutagenesis gave satisfactory results (10–25% mutants), we are now routinely using the method of Taylor et al. (1985), which has been described to produce mutations with high frequency with M13 single-stranded DNA. We have applied this method for single-stranded DNA from pEMBL plasmids and have obtained frequencies of mutations (taking mutations into account which are not described here) ranging from 20% to 90%. Mutagenesis with pEMBL has the advantage that DNA inserted into the

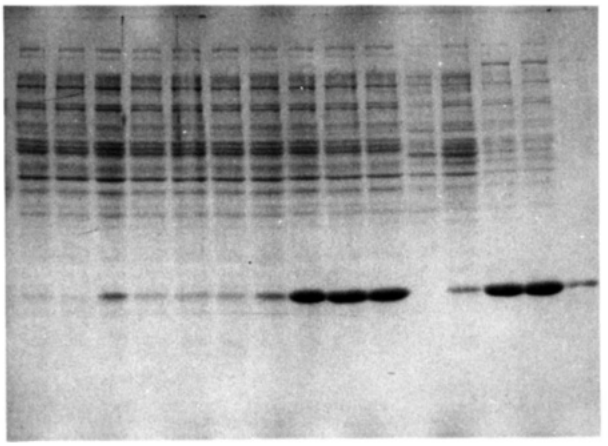

FIGURE 3: Comparison of adenylate kinase expression in *E. coli* cells containing different plasmid constructions. Crude cell extracts (0.5 mL) were prepared from 1-mL overnight cultures as described under Materials and Methods. From each of those 15 μL was applied to a 15% SDS–polyacrylamide gel (Laemmli, 1970) as indicated: lane 1, RRIΔM15; lane 2, RRIΔM15, "induced" with 50 μM IPTG; lane 3, RRIΔM15 (pFAK1); lanes 4–6, RRIΔM15 (pFAK1), induced with 50, 100, and 500 μM IPTG, respectively; lane 7, RRIΔM15 (pFAK11); lanes 8–10, RRIΔM15 (pFAK11), induced with 50, 100, and 500 μM IPTG, respectively; lane 11, CV2; lane 12, CV2 (pAK601); lane 13, CV2 (pEAK90); lane 14, CV2 (pEAK91); lane 15, CV2 (pEAK91), with 3 μL of crude extract applied. Enzyme activities in crude extracts were determined by the optical test for the reverse reaction of AK and are given in units/mL of cell culture. CV2 (lane 11) grew at 28 °C and all other clones at 37 °C.

Table I: Sequences of Oligonucleotides Used for Site-Directed Mutagenesis Aligned with the Sequence of the Coding Strand and the Derived Amino Acid Sequence of AKe in the Region of the Nucleotide Binding Loop[a]

5'ATT CTG CTT GGC GCT CCG GGC GCG GGG AAA GGG ACT CAG GCT CAG TTC 3'	
I L L G A P_9 G_{10} A G K_{13} G T Q A Q F	
3' CG CGC CCC GTT CCC TGA	Lys_{13} → Gln_{13}
3' G CGA GGC CAG CGC CCC T	Gly_{10} → Val_{10}
3' A CCG CGA GAC CCG CGC C	Pro_9 → Leu_9
3' CAAATAGCTAAAAAGTT	ClaI site at pos. -50

[a] Asterisks above the oligonucleotide sequence indicate the positions of the mismatches.

plasmid-type vector seems to be more stable than in a vector like bacteriophage M13 (Dente et al., 1983), and the use of a high copy number plasmid like pEMBL9 facilitates the preparation of double-stranded DNA for restriction analysis and the expression of protein (see above). In the case of adenylate kinase the same vector pEAK90 can be used for overexpression of the *adk* gene, which allows an easy screening of the enzyme activity of mutant clones in the crude extract. The method of Taylor et al. (1985) is also not restricted to DNA or bacterial strains with special genotypes.

We isolated the restriction fragments containing the desired mutation and introduced them into pEAK90 deleted for the according fragments. These clones were characterized by DNA sequencing of the complete recloned fragments as outlined under Materials and Methods to verify that no unexpected mutations had occurred. They were then transformed into the *E. coli* strain CV2, which has a chromosomal temperature-sensitive mutation and accordingly has a very low

Table II: Adenylate Kinase Activities of Crude Extracts[a]

strain (Ake plasmid)	activity (units/mL)
CV2	0.03
CV2 (wt)	57
CV2 (PL9)	18.3
CV2 (GV10)	27.6
CV2 (KQ13)	0.45

[a] Crude cell extracts were prepared from 1-mL overnight cultures and measured for AK activity in the forward reaction as described in Table I.

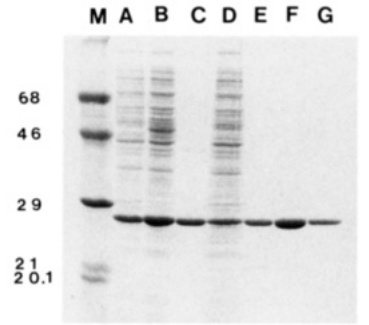

FIGURE 4: SDS–polyacrylamide gel electrophoretogram of crude extracts and purified proteins. Solutions were applied to a 15% SDS–polyacrylamide gel (Laemmli, 1970) and contained the following samples: lane M, marker proteins with molecular mass in kilodaltons (66, 45, 29, 21, 20.1); lane A, crude extract of KQ13; lane B, crude extract of GV10; lane C, GV10 after Blue Sepharose column; lane D, crude extract of PL9; lane E, PL9 after Blue Sepharose column; land F, PL9 after DEAE-cellulose column; lane G, wt after Blue Sepharose column.

background AKe activity (see Table II and Figure 3). The resulting clones were grown overnight and a soluble extract of cells was prepared. Figure 4 shows an SDS–polyacrylamide gel of the cell extract of mutant clones. It can be seen that expression of adenylate kinase is high for wild-type and mutant clones. Adenylate kinase activity was also measured in crude cell extracts and is indicated in Table II. Since the amount of protein is similar for wild-type and mutant proteins, the activity measurements of crude extracts are already an indication of the properties of mutants. AKe from the mutants PL9 and GV10 has a reduced activity, whereas the KQ13 mutation is nearly totally inactive. Thus the PL9 and GV10 mutant proteins were purified, whereas the KQ13 mutation was not further investigated.

Wild-type and mutant proteins were purified with Blue Sepharose as a first step. By eluting the proteins with a linear salt gradient instead of nucleotides as described Barzu et al. (1983), we obtain adenylate kinase that is free of nucleotides as judged by HPLC (see Materials and Methods). Wild-type adenylate kinase and the GV10 mutant were >98% pure as judged by SDS gel electrophoresis (Figure 4). PL9 protein was further purified by gradient elution on a DEAE-Sepharose column. Figure 4 shows the results of the purification. The PL9 mutant is estimated to be >95% pure after Blue Sepharose and >99% pure after the DEAE-Sepharose column.

Kinetic Analysis of Adenylate Kinase Mutants. The ability of the mutant proteins to catalyze the reaction

$$AMP + MgATP \rightarrow ADP + MgADP$$

was tested under steady-state conditions as outlined under Materials and Methods. Figure 5 shows as an example the data for the determination of the K_m for AMP of wild type and PL9 AKe. The data are plotted according to the method of Hanes (1923), and one can see that the intercept on the x axis which gives the K_m is vastly different for the two proteins.

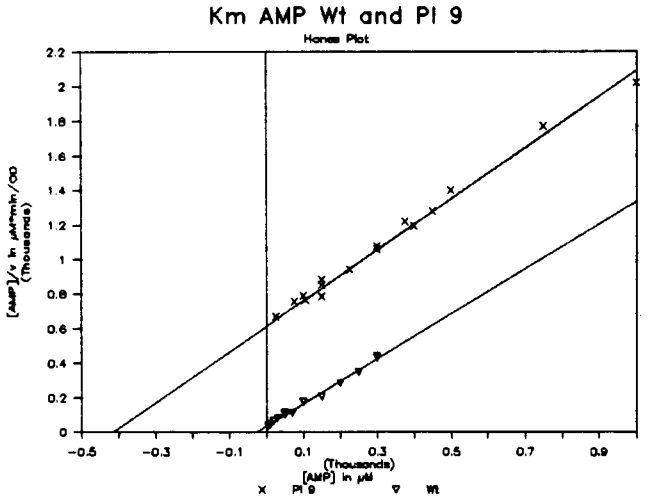

FIGURE 5: Hanes plot for the determination of K_m(AMP). The initial rates of the adenylate kinase forward reaction for PL9 and wild-type AKe were plotted according to Hanes (1923), taking directly the change of optical density with time as a measure of reaction velocity. The experimental data from wild type were corrected for different enzyme concentrations used in the two experiments. Experimental conditions were as outlined in Table III.

The slope of the plot that is used to calculate V_{max} is very similar. The results of all these plots are shown in Table III and compared to wild-type kinetic constants measured with the same system. The PL9 mutation shows a drastic increase in the K_m values for ATP as well as for AMP by a factor of about 15. The V_{max}, on the other hand, is only slightly decreased under the experimental conditions. Likewise the protein with glycine$_{10}$ exchanged for valine shows a 15-fold increase in the K_m for AMP and a smaller increase for the K_m of ATP by a factor of 7. The V_{max} value for AMP is reduced by a factor of 2 whereas for ATP it is down by a factor of 4.

One has to keep in mind that the determination of V_{max} values for one substrate is dependent on the concentration of the other. The K_m values for the mutant proteins are higher than the concentrations of the fixed substrates in nearly all cases. Since the fixed concentration is 300 μM for AMP and 1000 μM for ATP, one can easily see that the ATP site is not saturated for PL9 and GV10 and the AMP site is even less than half-saturated for the mutants when V_{max} and K_m are determined for the variable substrate. The V_{max} values determined for the variable substrate are therefore lower than the true V_{max}, which one would expect with complete saturation of the second site. Direct measurements of the true value for V_{max} for the mutants are not possible. If the concentration of AMP is increased in order to saturate its binding site, we observe an inhibition: competitive with regard to ATP and uncompetitive with regard to AMP. This inhibition is also seen for wild-type protein when AMP exceeds 300 μM (data not shown), which has also been described for AK1 (Rhoads & Lowenstein, 1968; Kuby et al., 1962) and pyrimidine nucleoside monophosphate kinase (Seagrave & Reyes, 1987). For this reason and to get an idea of the possible V_{max}, the measured $V_{max_{exp}}$ for the variable substrates are recalculated with the formula:

$$V_{max_{cor}} = V_{max_{exp}} f_{cor}$$

where the correction factor f_{cor} is given by

$$f_{cor} = ([S]_f + K_{m_f})/[S]_f$$

$[S]_f$ is the concentration of the fixed substrate at which the kinetic constants have been determined, and K_{m_f} is the value shown in Table III, determined under subsaturating conditions.

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

ATP as Variable Substrate				
enzyme	K_m(ATP) (μM)	V_{max}(ATP) (units/mg)	k_{cat}(ATP) (s^{-1})	k_{cat}/K_m-(ATP) (s^{-1} μM^{-1})
wt	71	780	305	4.3
PL9	1180	530	207	0.17
GV10	530	190	75	0.14
AMP as Variable Substrate				
enzyme	K_m(AMP) (μM)	V_{max}(AMP) (units/mg)	k_{cat}-(AMP) (s^{-1})	k_{cat}/K_m-(AMP) (s^{-1} μM^{-1})
wt	26	770	301	11.6
PL9	442	690	270	0.66
GV10	482	360	140	0.29

[a] The reaction medium contained, in 1-mL final volume, 100 mM Tris-HCl, 100 mM KCl, 2 mM $MgCl_2$, 10 units each of lactate dehydrogenase and pyruvate kinase, 200 μM phosphenolpyruvate, and 400 μM NADH. ATP was varied between 5 and 500 μM for wild type and between 50 and 1500 μM for mutants with AMP fixed to 300 μM in each case. AMP was varied between 5 and 300 μM for wild type and between 25 and 1000 μM for mutants with a fixed ATP concentration of 1000 μM. The reaction was started with 10 μL of enzyme solution diluted to the desired concentration (between 0.05 and 0.1 μg). For calculation of k_{cat} a mol wt of 23 500 was used.

Table IV: Corrected Values for Experimental Kinetic Data Extrapolated to Saturation for the Site of the Fixed Substrate As Outlined in the Text

ATP as Variable Substrate				
enzyme	f_{cor}	V_{max}(ATP) (units/mg)	k_{cat}(ATP) (s^{-1})	k_{cat}/K_m-(ATP) (s^{-1} μM^{-1})
wt	1.08	842	330	4.65
PL9	2.46	1300	509	0.43
GV10	2.61	503	197	0.37
AMP as Variable Substrate				
enzyme	f_{cor}	V_{max}(AMP) (units/mg)	k_{cat}-(AMP) (s^{-1})	k_{cat}/K_m-(AMP) (s^{-1} μM^{-1})
wt	1.07	824	322	12.4
PL9	2.18	1500	587	1.33
GV10	1.53	550	215	0.45

The corrected kinetic constants are shown in Table IV. The fact that the recalculated values for V_{max} are nearly the same for both substrates indicates the validity of this method for obtaining an approximation to the real V_{max} values. One can also see that as expected the correction factor for wild-type enzyme is close to unity, indicating that for wild-type AKe the two sites were saturated under the experimental conditions. One can see from the corrected values of V_{max} presented in Table IV that whatever the real V_{max} is for the mutated proteins, it is as high as or higher than for wild-type AKe. To rule out the possibility that the measured activity which underlies the production of ADP is not partly due to ATPase activity, the coupled assay was also done with 1 mM ATP and 300 μM AMP in the normal way and with 1 mM ATP alone with higher concentrations of wild-type and mutant proteins. These tests indicated that the adenylate kinase activity is at least 5×10^3 times higher than any ATPase activity.

NMR Measurements. For any type of mutation one would like to know whether the introduction of a new amino acid at a particular position in the polypeptide chain does or does not change the local or overall structure of the protein. NMR is well suited to answering such questions because it gives signals for all individual protons of the mutants, although in practice for proteins with molecular weights over, e.g., 8000 daltons, most of the individual resonances overlap. For the *E. coli*

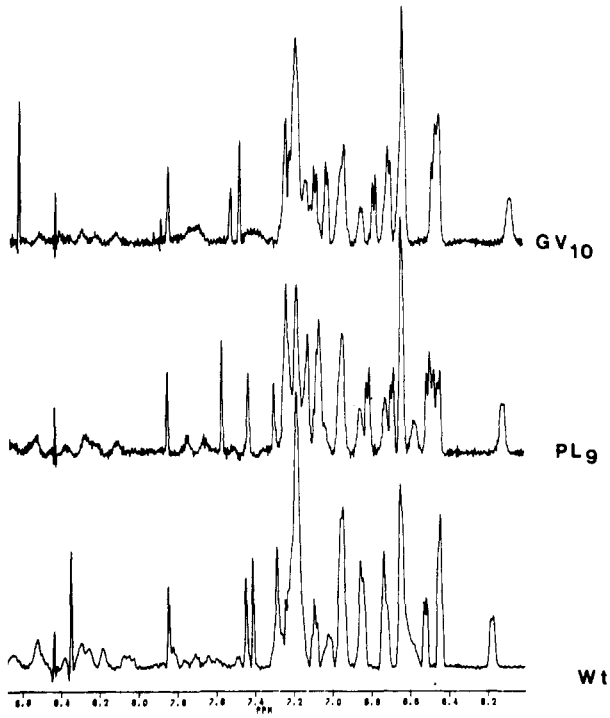

FIGURE 6: Proton NMR spectra of the aromatic side chains of *E. coli* adenylate kinase wild-type and mutant proteins: temperature, 300 K; buffer, 100 mM phosphate at pH 7.5; protein concentrations, 0.7 mM. An internal DSS (sodium 4,4-dimethyl-4-silapentane-1-sulfonate) reference was used for calculating chemical shifts in ppm. All spectra were digitally processed by Gaussian line sharpening.

adenylate kinase it was possible to identify most of the aromatic protons by using two-dimensional NMR spectroscopy (Bock et al., 1987). Here we prepared one-dimensional spectra of the wild-type and mutant proteins to get an idea of possible structural rearrangements of the protein due to the mutations. The high-field portions of the spectra are shown in Figure 6. One can see that many aromatic residues have changed their chemical shifts, thus indicating that a major rearrangement seems to have taken place. We have shown the spectrum of a fully active AKe mutant of *E. coli* where Tyr_{24} was changed to Phe_{24}. The 1-D spectrum of this mutant was identical with that of wild-type AKe in the aromatic region except that one tyrosine was missing and one additional phenylalanine residue appeared in the spectrum (Bock et al., 1987). This demonstrates that proton NMR of proteins is a valuable source of information about the structural integrity of mutant proteins. This helps one to analyze whether or not the amino acid exchange influences the folding of the protein and/or its catalytic activity. Although such information can be obtained in greater detail by X-ray crystallography, the NMR method consumes much less time and material.

Discussion

The importance of $lysine_{21}$ (which corresponds to Lys_{13} in *E. coli*) for catalysis in AK1 from rabbit muscle was proved by Tagaya et al. (1987), who could show that modification of this residue by adenosine diphosphopyridoxal with subsequent reduction inactivated the enzyme. Labeling was prevented by addition of ADP or ATP, which argues for the presence of Lys_{21} in the phosphate binding region. The exchange of the corresponding lysine with glutamine and glutamate in F_1-ATPase reduces the activity to 12% and 25% of normal (Parsonage et al., 1987). Fry et al. (1985) suggested from NMR measurements that the similarly located lysine in adenylate kinase may interact with the α-phosphate of ATP. Direct evidence for the localization of Lys_{21} from pig muscle AK1 came from a crystallographic study by Dreusicke and Schulz (1986), who found a bound sulfate ion occupying a phosphoryl group binding site making contact with Lys_{21}, as shown in Figure 1B. In AKe the mutation of this lysine residue by glutamine also severely reduces enzymatic activity. Whether the KQ13 protein is totally inactive or still has a very small residual activity cannot easily be determined because of the activity of the chromosomally encoded adenylate kinase from CV2. The mutant also does not bind to Blue Sepharose which is believed to mimic the ligand binding site on nucleotide binding enzymes (Thomson et al., 1975). We also do not know whether the KQ13 mutant has a native conformation. However, using crude extracts on a native polyacrylamide gel, we found that this protein runs distinctively faster that wild-type and other mutant proteins. The fact that large amounts of this protein are found in crude cellular extracts, see Figure 4, seems to indicate that the protein is not unfolded. If it were, one would expect the protein to be broken down by the bacterial proteases like La, which specifically recognizes unfolded proteins (Goff & Goldberg, 1985).

Our kinetic analysis showed that the initial rate constants measured for wild-type AK from *E. coli* in this work are comparable to those reported by Gilles et al. (1986) and are close to the data reported for adenylate kinase of yeast (Ito et al., 1980), which also is a member of the long AK family (Schulz et al., 1986). The mutations $Gly_{10} \rightarrow$ Val and $Pro_9 \rightarrow$ Leu in the nucleotide binding loop are slightly less active than wild-type AKe and lead to K_m values that are more typical of other adenylate kinases like the mammalian cytoplasmic AK1 type (Tomaselli & Noda, 1979).

Mutations of the Gly-X-Gly-X-X-Gly motif have also been described for other proteins like the β-subunit of F_1-ATPase and tRNA nucleotidyltransferase from *E. coli* (Zhu et al., 1986). The β-subunit of F_1-ATPase has the sequence Gly-Gly-Ala-Gly-Val-Gly-Lys-Thr (149–156), which is highly homologous to adenylate kinase. The overall high homology between these two enzymes has suggested a three-dimensional model of the β-subunit based on the crystal structure of adenylate kinase (Fry et al., 1986). The effect of replacing $alanine_{151}$ with the more bulky valine is to increase the K_M of ATP by a factor of 3 and the initial rate of uni-site hydrolysis slightly (Hsu et al., 1987). Thus the nature of the effects on the kinetics are very similar for the two proteins, although the magnitude of the effect on the binding of ATP is greater for adenylate kinase. Parsonage et al. 1987) have changed by site-directed mutagenesis the glycine residues 149 and 154 of the nucleotide binding loop of the β-subunit of F_1-ATPase, but the position of these residues is not analogous to the AKe mutations shown here.

It is known from X-ray crystallography that pig muscle AK1 can exist in at least two conformations depending on the crystallization conditions (Sachsenheimer & Schulz, 1977). The authors pointed out that the different crystal structures are not likely to be a consequence of molecular packing but rather reflect the intrinsic capacity of the molecule to undergo conformational changes. In the transition from crystal form A to crystal form B the most drastic spatial rearrangement takes place in the nucleotide binding loop 16–22, which corresponds to amino acids 8–14 in AKe, some of which have been mutated here. Further experiments of Pai et al. (1977) indicated that the two crystal forms may be related to different conformations resulting from the induced fit mechanism that was postulated for all phosphoryl-transferring enzymes by Koshland (1958) and Jencks (1975) in order to prevent hy-

drolysis as a side reaction. In support of this, the NMR spectra of wild-type adenylate kinase from *E. coli* show a structural rearrangement when the enzyme is saturated with the substrates ATP or AMP (Bock et al., unpublished results), as has been found for a human muscle AK1 inhibitor complex (Kalbitzer et al., 1982).

The kinetic consequences for an enzyme conforming to the induced fit mechanism were described by Fersht (1985). The enzyme exists in an inactive and an active conformation and in a free and a substrate-bound form. The equilibria between these forms are described by their appropriate dissociation constants $K = [E_{act}]/[E_{in}]$ and $K' = [E_{act}S]/[E_{in}S]$. We assume that $K \ll 1$ and $K' \gg 1$, which means that nearly all free enzyme is in the inactive form and nearly all substrate-bound enzyme in the active form. Under these circumstances the measured K_m value is increased by the factor $1/K$ as compared to the K_m value that would be found without an induced fit mechanism for the enzyme. Under the same conditions the k_{cat} value would not be altered by the induced fit. Using these considerations, we assume from the kinetic and structural data on the mutants PL9 and GV10 that these proteins have an inactive conformation which is believed to be different from the inactive form of wild-type AKe. These proteins would have a higher equilibrium constant K for the conversion between the active and inactive enzyme. As a result the K_m value would increase and V_{max} would not be affected. We do not regard this model as the only one able to explain the obtained data, but it appears to be the most plausible. Thus the nucleotide binding loop of adenylate kinase seems to be important not only for the binding of substrates but also because of its great flexibility, as an element in structural rearrangements that are necessary for the function of the enzyme.

The glycine-rich loop of adenylate kinase is a well conserved structural unit that can be found in all guanine nucleotide and many adenine nucleotide binding enzymes. A great number of human tumors have been described that contain mutations in positions 12 and 13 of the N-, K-, and Ha-*ras* genes that encode the protein p21. It is now well established that the mutation of the p21 protein is involved in the development of these tumors. The structural and biochemical consequences of the mutation of glycine-12/13 are, however, not well understood, except that the GTPase activity of the protein in vitro is reduced by a factor of approximately 10. We have recently shown that the mode of GDP binding and the overall structure of the native and mutated proteins are very similar, except that the release of bound GDP and GTP is much slower and their binding constants are higher in $Gly_{12} \rightarrow$ Val and $Gly_{12} \rightarrow$ Arg mutations (John et al., unpublished results). Further insight into the structural alterations resulting from these mutations in the loop must however await the elucidation of the three-dimensional structure of p21.

In contrast to p21, the three-dimensional structure of adenylate kinase has been determined, and the refined structure of the nucleotide binding loop is shown in Figure 1 [taken from Dreusicke and Schulz (1986)]. It has been stated that the backbone dihedral angles of some residues in the adenylate kinase loop are forbidden for amino acids with side chains, which would explain the apparent alteration in 3-D structure as a consequence of structural strain, as evidenced by the significant changes in the NMR spectrum. In contrast to this, we have found that the mutation in the nucleotide binding loop of p21 proteins has no apparent effect on the conformation of the protein, again as evidenced by proton NMR (Schlichting et al., unpublished results). This seems to indicate that, although the so-called nucleotide binding loop is contained in many nucleotide binding proteins and may be situated close to the phosphate residues in the binding site of all these proteins, its flexibility or functionality may be different between them.

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