Adenylate Kinase of *Escherichia coli*: Evidence for a Functional Interaction in Phospholipid Synthesis†

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ABSTRACT: Previous genetic and biochemical experiments have suggested that the adenylate kinase of *Escherichia coli* may be directly involved in phospholipid synthesis through formation of a complex with *sn*-glycerol-3-phosphate acyltransferase, the membrane-bound enzyme that catalyzes the first step in phospholipid synthesis. In this paper we report direct experiments to test this hypothesis. A mutation within the adenylate kinase structural gene is described that results in a temperature-sensitive phospholipid synthesis (assayed in vivo) and a temperature-sensitive acyltransferase. The adenylate kinase activity of this strain is only minimally altered either in vitro or [as assayed by adenosine 5′-triphosphate (ATP) levels] in vivo. This result demonstrates that the inhibition of phospholipid synthesis is not the result of reduced ATP levels. We report the purification of *E. coli* adenylate kinase to homogeneity and find that the addition of homogeneous wild-type adenylate kinase to membranes containing a mutationally altered temperature-sensitive acyltransferase results in thermal stabilization of the acyltransferase activity. Ovalbumin has no such protective effect. Purified *E. coli* inner membranes contain several proteins that are precipitated by addition of anti adenylate kinase antibody to detergent-solubilized membranes.

Mutants of *Escherichia coli* K-12 that produce an altered adenylate kinase have a complex phenotype (Cronan, 1978). These strains (formerly called *plsA*; now called *adk*) were isolated as being temperature-sensitive mutants defective in phospholipid synthesis (Cronan et al., 1970). It was later shown that *adk* mutants are also temperature sensitive in the synthesis of other macromolecules (RNA, DNA, and protein) (Glaser et al., 1973). The phospholipid synthetic defect in these strains was localized to a temperature-sensitive *sn*-glycerol-3-phosphate acyltransferase (Cronan et al., 1970; Ray et al., 1970). This inner membrane bound enzyme catalyzes the first committed step in phospholipid synthesis (Cronan, 1978). Further work showed that a second enzyme, the cytoplasmic adenylate kinase, is also temperature sensitive (Glaser et al., 1975). Genetic analysis showed that a single mutation at one genetic locus caused the thermolability of both adenylate kinase and the *sn*-glycerol-3-phosphate acyltransferase (Cronan & Godson, 1972; Esmon et al., 1980), and it was concluded that the mutational lesion occurred in the structural gene, *adk*, for adenylate kinase (Glaser et al., 1975). The defective adenylate kinase explained the decreased levels of ATP,[1] the increased levels of AMP, and the inhibition of macromolecule synthesis detected in several of the *adk* mutants at nonpermissive temperatures (Glaser et al., 1975). It did not, however, explain the decrease in phospholipid synthesis at nonpermissive temperatures because at semipermissive temperatures nucleic acid synthesis (which is very sensitive to ATP levels) proceeded normally whereas phospholipid synthesis [which is quite insensitive to ATP levels (Hennen et al., 1978)] was inhibited (Ray et al., 1976).

The most straightforward hypothesis able to explain these data is that put forward by Glaser and co-workers (Glaser et al., 1973, 1975; Esmon et al., 1980). This hypothesis predicts that in addition to its role in the production of ATP in the cytosol, adenylate kinase functionally interacts with the membrane-bound *sn*-glycerol-3-phosphate acyltransferase. Thus, a single mutational event could result in thermolability of both enzyme activities and thus explain the preferential effects on phospholipid synthesis observed at semipermissive temperatures. Moreover, this hypothesis provides a rationale for the stimulation of the acyltransferase by ATP (Merlie & Pizer, 1973; Rock et al., 1981b), a substrate of adenylate kinase. In this paper we report experiments testing this hypothesis.

Materials and Methods

Bacterial Strains and Media. Strains 8, CV15, and CV31 were previously described (Cronan et al., 1970; Cronan & Godson, 1972). Strains SEG5, SEG10, and SEG11 were constructed by transduction of strain X478 to *purE*$^+$ *adk*$^-$ with phage P1*vir* as described by Cronan & Godson (1972). Strains 8 and X478 are *adk*$^+$ strains whereas strains CV15 and SEG5 carry the *adk-15* allele. The *adk-31* allele is carried by strains SEG10, SEG11, and CV31.

The main source of material for enzyme purification was frozen cell pastes of *E. coli* B (Grain Processing Corp., Muscatine, IA). Adenylate kinase was also purified from strain 8. Strain VL1A/pVL1 was the generous gift of Dr. Robert Bell (Lightner et al., 1980).

The medium for the [^{35}S]methionine labeling of strain VL1A/pVL1 was as follows (per liter): 13.6 g of KH_2PO_4, 2.0 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4$, and 50 μg of $FeSO_4 \cdot 7H_2O$. The pH was adjusted to 7.2 with KOH. To 200 mL of the autoclaved medium was added 5 mL of 1 mg/mL thiamin hydrochloride, 50 μg/mL of the L isomers of arginine, histidine, aspartic acid, glycine, leucine, isoleucine, lysine, proline, threonine, and serine, and 25 μg/mL each of phenylalanine and tyrosine. The medium for $^{32}PO_4$ labeling was (per liter) as follows: peptone, 10 g; NaCl, 5 g; glucose, 1 g; Tris-HCl (pH 7.5), 1 mmol.

$^{32}PO_4$ Labeling of plsA Stains in Vivo. Overnight cultures were diluted to 5 × 10^7 cells/mL. When a density of 2 × 10^8

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; AMP, adenosine 5′-monophosphate; Tris, tris(hydroxymethyl)aminomethane; PEI, polyethylenimine; ADP, adenosine 5′-diphosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NADP, nicotinamide adenine dinucleotide phosphate; $NaDodSO_4$, sodium dodecyl sulfate.

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cells/mL was reached, 10 mL of the culture was removed to a side-arm flask to monitor growth, and a 2-mL sample was placed in a small flask containing 60 μCi of $^{32}PO_4$ (carrier free). At each of the indicated time points, 0.1 mL of the culture was withdrawn and mixed with 0.1 mL of ice-cold 2 M formic acid for ATP determination. At the same time another 0.1-mL sample of the culture was pipetted into a tube containing 0.75 mL of $CHCl_3/CH_3OH$ (1:2 v/v) and 0.1 mL of a stationary-phase culture of *E. coli* (as carrier). These samples were used for RNA and phospholipid determinations as described by Kaimura-Kuroda et al. (1980). ATP concentrations were determined by chromatography on PEI–cellulose plates (Holmes & Singer, 1973).

Membrane Preparations. Inner membranes were prepared by equilibrium sucrose density gradient centrifugation as described by Rock et al. (1981a) and stored at −20 °C at a concentration of 10–20 mg of protein/mL in 20 mM potassium phosphate (pH 7.5) containing 10 mM $MgCl_2$. Cell envelopes (which contain both inner and outer membranes) were prepared and stored in the same manner, except that the sucrose gradient step was omitted and the membranes were washed once with the potassium phosphate–$MgCl_2$ buffer. Membrane protein concentrations were determined by the microbiuret assay (Munkres & Richards, 1965).

Enzyme Assays. Adenylate kinase activity was assayed by conversion of ADP to ATP. In most instances a spectrophotometric assay was used in which the generation of ATP from ADP was coupled to the production of NADPH through hexokinase and glucose-6-phosphate dehydrogenase. The assay mixture (0.5 mL) contained the following: Tris-HCl buffer (pH 7.5), 0.1 M; KCl, 0.1 M; glucose, 0.27 mM; $MgCl_2$, 2 mM; 2-mercaptoethanol, 21.3 mM; NADP, 0.5 mM; hexokinase, 47.5 IU; glucose-6-phosphate dehydrogenase, 10.5 IU; ADP, 2 mM. The assay was run at 23 °C and monitored at 340 nm. Identical values were given by the thin-layer chromatographic assay of Holmes & Singer (1973) using [8-^{14}C]ADP (0.2 Ci/mol). A unit of kinase activity is defined as the formation of 1 μmol of product/min.

Acyltransferase activity was assayed as described by Ray & Cronan (1975). A unit of acyltransferase activity is defined as 1 nmol of product formed/min.

$NaDodSO_4$ Electrophoresis. The system used was that of Laemmli (1970). The slab gels were of 10 or 15% polyacrylamide [ratio of acrylamide to bis(acrylamide) = 16:1], and the samples were boiled in Laemmli's sample buffer for 3–5 min before loading. Staining and destaining were done as previously described (Rock & Cronan, 1979).

Immunological Procedures. Antibodies to adenylate kinase (1 mg followed by two 0.2-mg booster injections) were raised in New Zealand rabbits as described by Livingston (1974). One of three rabbits produced anti adenylate kinase antibodies as assayed by immunoprecipitation and inhibition of *E. coli* adenylate kinase activity. Purified IgG was prepared from the serum by the method of Livingston (1974) as modified by Broome & Gilbert (1978). Anti adenylate kinase IgG fractions completely inhibited the activity of purified preparations of *E. coli* adenylate kinase. Antibody precipitation was accomplished by the use of cells of *Staphylococcus aureus* (Pansorbin from Calbiochem) (Kessler, 1975).

Isoelectric Focusing. Isoelectric focusing was performed in 7.5% acrylamide tube gels containing 1% Biolyte 4/6, 1% Biolyte 5/7, and 0.5% Biolyte 3/10. The ratio of acrylamide: bis(acrylamide) was 39:1. The buffer solutions were 0.06 N $(NH_4)_2SO_4$ (positive upper reservoir) and 0.04 N NaOH–0.2 N $Ca(OH)_2$ (negative reservoir). After preelectrophoresis, the gels were loaded with the samples at the acidic end and run for 20 h. The pH gradient was measured by sectioning a gel run in parallel. The gels were stained and destained as described above.

Other Protein Analytical Techniques. Equilibrium sedimentation was performed by using the modified method of Bothwell et al. (1978) as described by Rock & Cronan (1979) except that the sample was centrifuged at 10 psi (about 58 000 rpm) for 21.5 h in a Beckman Airfuge. The $\bar{v}$ of adenylate kinase was calculated from the amino acid composition (Goelz, 1979). N termini were determined by using the method of Weiner et al. (1972).

Protein concentration was determined spectrophotometrically (Warberg & Christian, 1941; Murphy & Kies, 1960) or by a microbiuret procedure (Munkres & Richards, 1965). In the adenylate kinase purification, ADP and AMP interfered with the aforementioned assays, as well as with other common protein assays. Trichloroacetic acid precipitation (Kunitz, 1952) was used to measure protein concentrations in fractions eluted from the affinity columns. All protein assays were standardized against bovine serum albumin.

Purification of Adenylate Kinase. (A) Acid and Ammonium Sulfate Fractionations. In a typical preparation, 200 g of a frozen cell paste of *E. coli* B (equivalent results were obtained with *E. coli* K-12) was resuspended in 400 mL of TM buffer. TM buffer was a 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$. The suspension was passed through a French pressure cell (20 000 psi) and freed of unbroken cells and large debris by centrifugation at 12000*g* for 40 min (cell disruption and all subsequent purification steps were performed at 0–4 °C). To the crude extract was added 0.1 volume of a 30% (w/v) streptomycin sulfate solution over 10 min with continual stirring. After the solution was further stirred (45 min), it was centrifuged at 12000*g* for 20 min.

The supernatant was adjusted to pH 4.0 with dilute HCl, stirred for 10 min, neutralized to pH 7.0 with 1 N NaOH, and centrifuged at 12000*g* for 10 min. The supernatant was adjusted to 55% saturation with solid ammonium sulfate and equilibrated for 1.5–2 h. The mixture was centrifuged (12000*g* for 15 min), and the supernatant was adjusted to 80% saturation with solid ammonium sulfate as above. The pellet was suspended and dialyzed overnight in TM buffer.

(B) Blue Sepharose Chromatography. A sample (1.1 g of protein in a volume of 20 mL) of the 55–80% ammonium sulfate fraction in TM buffer was applied to a 24 × 1 cm column of Blue Sepharose (Pharmacia), and the column was eluted with 400 mL of 50 mM Tris-HCl (pH 7.5) to remove unbound protein. Adenylate kinase was eluted with 200 mL of the same buffer containing 0.5 mM ADP, and 25-mL fractions were collected. The fractions having enzymatic activity (90% of the activity was found in the first 50 mL of the ADP eluate) were pooled, transferred to dialysis tubing, and concentrated 10-fold by placing the tubing in dry poly(ethylene glycol) 6000 for 2–4 h at 4 °C with shaking.

(C) Gel Filtration. A 2.5-mL sample of the concentrated eluate from the Blue Sepharose column was applied to a 2.5 × 100 cm column of Sephacryl S-200 (Pharmacia) and eluted (5-mL fractions) with 10 mM Tris-HCl (pH 7.5). The fractions having adenylate kinase activity were examined by $NaDodSO_4$ gel electrophoresis, and those fractions (about 40%) in which adenylate kinase was the only protein were pooled.

(D) Alternate Procedure. The active fractions eluted from the Blue Sepharose column were pooled and dialyzed first against 0.5 M KCl in 50 mM Tris-HCl (pH 7.5) and then

Table I: Purification of Adenylate Kinase from *E. coli*[a]

step	activity (units)	protein (mg)	sp act. (units/mg)	purification (x-fold)	yield (%)
streptomycin sulfate	22400	25000	0.9	(1.0)	(100)
acid precipitation	19000	17300	1.1	1.2	85
ammonium sulfate (55–80%)	14600	5500	2.65	2.9	65
Blue Sepharose	11200	100	112.0	124	50
gel filtration	6720	7	960.0	1067	30

[a] A purification from 200 g of a frozen paste of *E. coli* B is given.

against the same buffer lacking KCl. This dialyzate was then loaded on a column (24 × 1 cm) of blue dextran 2000 (Pharmacia) bound to Sepharose (Ryan & Vestling, 1974). The column was eluted as described above for the Blue Sepharose column except that the eluting nucleotide was 2 mM AMP. $NaDodSO_4$ gel electrophoresis showed that about 80% of the fractions having adenylate kinase activity contained only this protein. The blue dextran column was used because AMP failed to elute adenylate kinase from Blue Sepharose columns.

Comments on the Purification. The purification is summarized in Table I. The major purification was that achieved by binding adenylate kinase to Blue Sepharose and elution with ADP. Over 85% of the activity applied to the column was eluted with ADP, and a 40–50-fold purification resulted. *E. coli* adenylate kinase was quite stable if stored in the presence of salt concentrations >50 mM. Under these conditions little loss of activity was seen at 4 °C for 2–3 weeks or at −20 °C for 3 months.

Our preparations appeared homogeneous after a 1000-fold purification by the following criteria: (i) $NaDodSO_4$ gel electrophoresis (Figure 5), (ii) isoelectric focusing (Figure 5), and (iii) N-terminal amino acid analysis (only methionine was found). A few preparations were also found homogeneous by gel electrophoresis at alkaline pH (the $NaDodSO_4$ gel system lacking detergent).

Physical Properties of Adenylate Kinase. Upon electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulfate, adenylate kinase had the mobility of a protein of M_r 27 500. We have intermittently observed the appearance of higher molecular weight forms of adenylate kinase upon storage and/or electrophoresis of homogeneous kinase preparations. We have tentatively identified these forms as aggregates (dimers and trimers) by the following criteria. (i) The aggregates ran on $NaDodSO_4$ gels with apparent molecular weights that were multiples (dimers and trimers) of the monomeric molecular weight. (ii) When the trimer band was eluted from the gel and reelectrophoresed, monomer, dimer, and trimer bands were again formed. (iii) The putative dimer band was eluted from the gel and shown to have significant (albeit low) adenylate kinase activity. (iv) Isoelectric focusing showed that all three forms had the same isoelectric point (pH 5.1). Although treatment of the gel samples with urea, reducing agents, increased $NaDodSO_4$ concentrations, or prolonged heating did not eliminate aggregation, preliminary results suggested that treatment with guanidine hydrochloride did disaggregate these higher molecular weight forms as assayed by gel filtration.

Gel filtration gave a molecular weight of 26 500, indicating that the active form of adenylate kinase was the monomer (Goelz, 1979). Sedimentation equilibrium gave a molecular weight of 26 000. Since the gel filtration experiments (which assume a globular shape) gave a result very similar to that

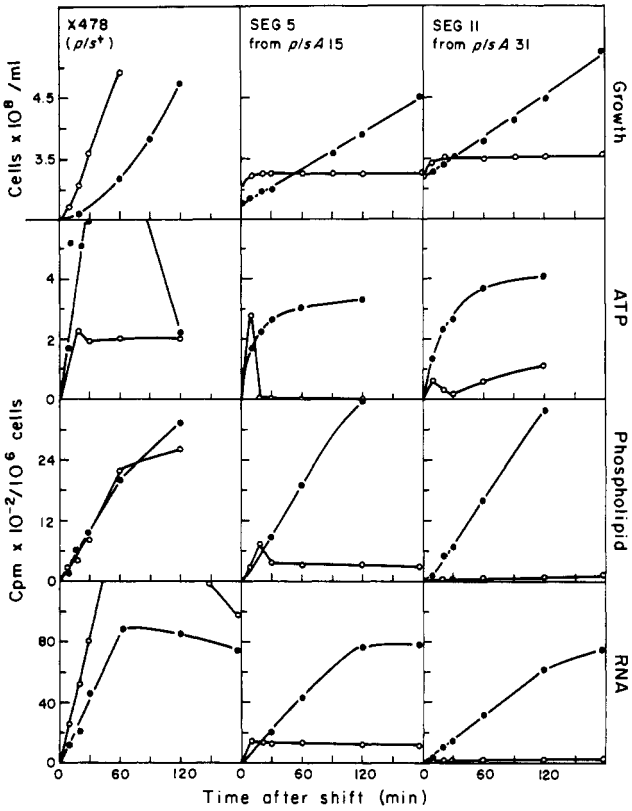

FIGURE 1: Phospholipid, RNA, and ATP synthesis in *adk* strains. Samples (2 mL) of cultures growing in the early log phase (2 × 10^8 cells/mL) at 25 °C were pipetted into flasks containing 60 μCi of $^{32}P_i$ at either 25 or 40 °C. At the times indicated, 100-μL samples were removed from each flask and processed for incorporation of ^{32}P into phospholipid, RNA, and ATP as described under Materials and Methods. The values are corrected for growth of the cultures during the experiment. Symbols: (●) 25 °C cultures and (○) 40 °C cultures.

of sedimentation equilibrium, the adenylate kinase of *E. coli* must be a globular protein. The Stokes radius found for the protein was about 24 Å (Goelz, 1979). Preliminary results indicated that the amino acid composition of *E. coli* adenylate kinase is not remarkable (Goelz, 1979).

Results

In Vivo Analysis of adk Mutants. Previous workers had shown that strain CV15, which carries the *adk-15* allele, possessed both a thermolabile adenylate kinase and a thermolabile *sn*-glycerol-3-phosphate acyltransferase (Glaser et al., 1975). In vivo studies with this strain demonstrated the inhibition of synthesis of phospholipid, ATP, nucleic acid, and protein at nonpermissive temperatures (Cronan & Godson, 1972; Glaser et al., 1973, 1975; Ray et al., 1976). We constructed a set of isogenic strains, X478, SEG5, and SEG11, that carried the *adk*$^+$, *adk-15*, and *adk-31* alleles, respectively. The *adk-31* allele was that of strain CV31, an *adk* strain previously shown to have a temperature-sensitive *sn*-glycerol-3-phosphate acyltransferase (Ray et al., 1970).

In agreement with earlier results with strain CV15, we found that SEG5, a strain carrying the same *adk* allele as CV15, was extremely temperature sensitive. Within 30 min after shift from 25 to 42 °C, phospholipid synthesis, RNA synthesis, and growth all were severely inhibited, and the intracellular level of ATP had decreased to <1% of the normal level at 42 °C (Figure 1). However, a parallel experiment with strain SEG11 gave a very different result. Although macromolecular synthesis was affected in a similar manner as in SEG5, the level of ATP reached 2 h after temperature shift was still quite high, about half of the level of the wild-type strain at 42 °C

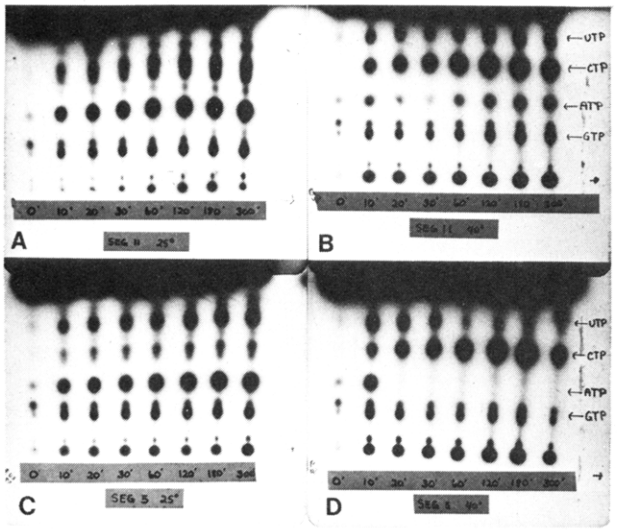

FIGURE 2: Pattern of incorporation of $^{32}P_i$ into nucleoside triphosphates. Strains SEG5 and SEG11 were grown and labeled with $^{32}P_i$ as described in Figure 1. The nucleotides were separated on PEI–cellulose thin-layer chromatograms, and the chromatograms were autoradiographed for 24 h. Panels A and B are of strain SEG11 labeled at 25 or 40 °C, respectively. Panels C and D are of strain SEG5 labeled at 25 or 40 °C, respectively. The compounds and the times of sampling (min) are given on the figure.

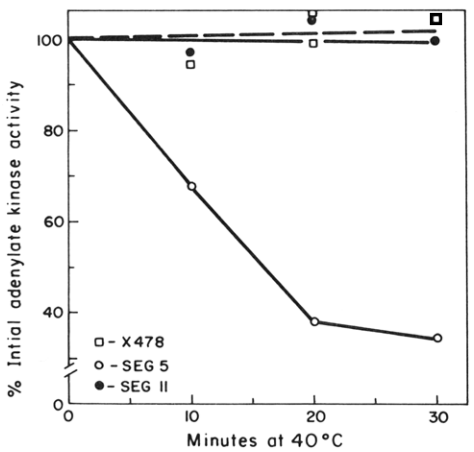

FIGURE 3: Thermolability of the adenylate kinases from strains X478, SEG5, and SEG11. Samples (40 μL) of cell-free extracts (40 mg of protein/mL) of the three strains were heated at 40 °C. At the times indicated 5-μL samples were removed to 0 °C and stored on ice until all samples had been taken. The samples were then assayed at 25 °C. The initial specific activities (units/mg of protein) of the extracts were as follows: strain X478 (□), 350; strain SEG5 (○), 60; strain SEG11 (●), 70.

(Figure 1). The unusual kinetics of ^{32}P incorporation into ATP in the *adk-31* strains were unique to ATP; the other ribonucleotide triphosphates monotonically reached a plateau level about 60 min after temperature shift (Figure 2). Similar results were obtained with strain CV31 and another transductant carrying the *adk-31* allele, strain SEG10.

Properties of the adk-31 Adenylate Kinase. The unusual behavior of the ATP pool of strain SEG11 in comparison to strain SEG5 and another *adk* mutant, strain CR341T28 (Glaser et al., 1975), suggested that the properties of the adenylate kinase from the *adk-31* strains should differ markedly from those of the other *adk* strains. We therefore examined the thermolability of the adenylate kinases of strains X478, SEG5, and SEG11 (Figure 3). As expected, the enzyme from strain SEG5 was much more thermolabile than the wild-type (strain X478) enzyme. However, the enzyme of

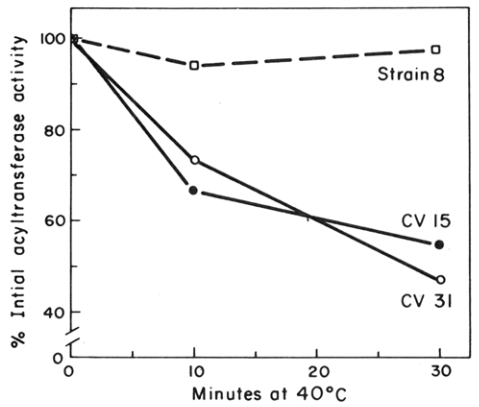

FIGURE 4: Thermolability of the acyltransferases of strains carrying the *adk-15* and *adk-31* alleles. Samples (40 μL) of purified cell envelopes (~25 mg of protein/mL) were incubated at 40 °C for the times indicated and then stored at 0 °C until assay. When all samples had been taken, the samples were assayed for acyltransferase activity at 25 °C. The initial (unheated) values (units/mg of protein) were as follows: strain 8 (□), 3.5; strain CV15 (●), 2.5; strain CV31 (○), 3.0. Similar results were obtained for other strains carrying the *adk-15* or *adk-31* alleles (Table II).

strain SEG11 was not abnormally thermolabile, and, in a number of experiments, the adenylate kinases of strains carrying the *adk-31* allele were somewhat less temperature sensitive than the wild-type enzyme. However, the thermostability of the SEG11 adenylate kinase did not extend to the acyltransferase; the acyltransferase activities of strains carrying this *adk* allele were as thermolabile as those of *adk-15* strains (Figure 4; Ray et al., 1970). From these data we conclude that the differing ATP pools of strains SEG5 and SEG11 can be attributed to differences in the type of alteration of the adenylate kinases of the two strains. Thus it follows that the inhibition of phospholipid synthesis observed in strain SEG11 cannot be due to the loss of adenylate kinase activity per se (i.e., a deficiency in the supply of ATP).

Genetic analyses clearly demonstrate that strain CV31 is an *adk* mutant (Cronan & Godson, 1972; Esmon et al., 1980). However, the adenylate kinases from strains carrying the *adk-31* allele were not abnormally thermolabile. To obtain biochemical evidence that *adk-31* strains produced an altered adenylate kinase, we attempted to purify the enzyme from strain SEG11. Unfortunately, neither the adenylate kinase from strain SEG11 nor that from strain SEG5 could be purified by the scheme developed for the wild-type enzyme. The mutant enzymes differed from the wild-type enzyme in a number of properties. First, crude supernatants of mutant cells (grown at 25 °C) had only about 20% of the adenylate kinase activity found in comparable supernatants of wild-type cells. Second, acid treatment (pH 4) of the mutant extracts resulted in a major loss (ca. 50%) of the original activity whereas the wild-type enzyme is stable under these conditions (Materials and Methods). Furthermore, the mutant enzymes precipitated at abnormally low ammonium sulfate concentrations. The ammonium sulfate concentration needed to precipitate 80% of either of the mutant enzymes was only half that required for the wild-type enzyme. The most striking difference, however, was the fact that neither of the mutant adenylate kinases could be eluted from Blue Sepharose with ADP. ADP concentrations of up to 10 mM were completely ineffective in eluting the mutant enzymes whereas 0.5 mM ADP quantitatively eluted the wild-type enzyme (Materials and Methods). The mutant enzymes could be eluted from Blue Sepharose with 0.5 M KSCN (or less effectively with 2 M NaCl) with some retention of enzymatic activity. $NaDodSO_4$ gel

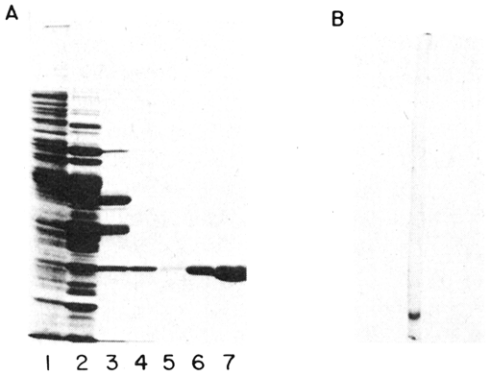

FIGURE 5: Purity of adenylate kinase assayed by $NaDodSO_4$ electrophoresis and isoelectric focusing. Panel A shows $NaDodSO_4$–polyacrylamide gel electrophoretograms of adenylate kinase at various stages of purification. The gel was of 10% acrylamide and was stained with Coomassie Blue. The samples were as follows: lane 1, supernatant from the streptomycin precipitation; lane 2, 55–80% $(NH_4)_2SO_4$ pellet; lane 3, the first active fraction from the Blue Sepharose column (Figure 1) after concentration; lane 4, a peak fraction from a Sephadex G-75 column; lanes 5–7, fractions across the activity peak of a Sephacryl-200 column. Panel B shows the isoelectric focusing gel of adenylate kinase. Approximately 20 μg of homogeneous adenylate kinase (prepared as in Table I) was focused on a tube gel as described in the text.

electrophoresis of these eluates showed a protein band with a mobility identical with that of wild-type adenylate kinase, suggesting that the mutant enzymes have the same molecular weight in the presence of $NaDodSO_4$ as the wild-type enzyme (data not shown).

The inapplicability of our purification method (Table I) to the mutant adenylate kinases and the lability of the mutant enzymes precluded their purification. However, the altered purification behavior of both mutant enzymes is consistent with previous evidence that both the *adk-15* and *adk-31* mutants encode a structurally altered adenylate kinase (Glaser et al., 1975; Ray et al., 1976).

In Vitro Evidence for Adenylate Kinase–Acyltransferase Interaction. To test the adenylate kinase–acyltransferase interaction predicted from the in vivo experiments, we have purified the adenylate kinase of *E. coli* to homogeneity and tested its effect on the acyltransferase. Although a number of laboratories have extensively purified *E. coli* adenylate kinase (Cousin & Buttin, 1969; Theze & Margarita, 1972; Holmes & Singer, 1973), purification to homogeneity had not been reported. We purified this enzyme to homogeneity by a procedure (Table I) utilizing only two column steps, neither requiring gradient elution. The purified enzyme appeared homogeneous by electrophoresis under denaturing conditions and isoelectric focusing (Figure 5) and was prepared in good yield (Table I). We have used these preparations to attempt to overcome the thermolability of the mutant acyltransferases of strains SEG5, SEG10, and SEG11 in vitro.

Effect of Purified Wild-Type Adenylate Kinase on the Mutant Acyltransferases. Cell envelopes prepared from strains X478, SEG5, SEG10, and SEG11 were incubated with homogeneous wild-type adenylate kinase for 3 days at 4 °C. Parallel samples of the same membrane preparation were incubated in the presence of ovalbumin or without added protein. Part of each mixture was then heated to partially inactivate the mutant acyltransferases and subsequently assayed for acyltransferase activity in parallel with an unheated sample (Table II).

Surprisingly, the unheated control samples containing added adenylate kinase had somewhat higher acyltransferase activities than the samples incubated without protein or with ovalbumin (Table II). However, the most remarkable result was that the acyltransferase activities of those samples containing wild-type adenylate kinase were much less thermolabile than the ovalbumin-treated or untreated samples. The acyltransferase activities of strains SEG5 and SEG11 showed the most pronounced effect although the effect on the acyltransferase of strain SEG10 was also greater than the ovalbumin control (Table II).

Table II: Modulation of Thermolability of *adk* Mutant Acyltransferases by Adenylate Kinase

strain	heated at 42 °C	acyltransferase activity (units/mg of protein) with: no added protein	ovalbumin added	adenylate kinase added
X478	–	3.1	3.4	3.8
X478	+	2.9	2.5	2.9
SEG5	–	2.2	2.5	2.9
SEG5	+	1.5	1.8	2.9
SEG10	–	2.0	1.6	2.5
SEG10	+	0.7	0.5	1.4
SEG11	–	2.8	2.7	2.9
SEG11	+	1.5	1.4	2.9

[a] Previously frozen cell envelopes (160 μg of protein) prepared from strains X478, SEG5, SEG10, and SEG11 were mixed with either 144 ng of homogeneous adenylate kinase from *E. coli* B or 144 ng of ovalbumin. The total volume was 18 μL. These samples and a sample containing cell envelopes without added protein were incubated at 4 °C for 3 days. A portion (4.5 μL) of each sample was then heated at 42 °C for 20 min and placed on ice. These heated samples were then assayed for acyltransferase activity in parallel with the unheated samples.

It should be noted that the ability of added adenylate kinase to overcome the thermolability of the acyltransferase of the *adk* mutants was enhanced when the membrane suspension had been frozen at −20 °C and thawed at room temperature before incubation with adenylate kinase at 4 °C. In addition, the thermolability of the mutant acyltransferases (but not of the wild-type acyltransferase) was also accentuated by freezing and thawing the membrane suspension before use.

Cellular Location of Adenylate Kinase. In disrupted preparations of *E. coli* most of the adenylate kinase activity was found in the cytosolic fraction. Thoroughly washed membrane fractions contained (at most) only a few percent of the enzyme activity. However, our ability to overcome the thermolability of the acyltransferase by incubation with normal adenylate kinase (Table II) indicated that inner membranes should contain some adenylate kinase in a complex form largely inactive in the adenylate kinase reaction.

Our first experiments using micro Ouchterlony plates showed that the amount of membrane-associated antigen reacting with anti adenylate kinase was quite large, comparable in staining intensity to the mass of soluble kinase (Goelz, 1979). However, if this antigen was adenylate kinase, the kinase was largely (>99%) inactive and from the shape and location of the precipitin lines in a complex of roughly the same molecular weight as IgG (~160 000). The rate of diffusion of the membrane-associated antigens was not altered by treatment of the membranes with 0.1 or 0.2% Triton X-100, a procedure that solubilizes the inner membrane proteins of *E. coli* (Schnaitman, 1971). Membranes from *adk* mutants contained multiple forms of antigen, suggesting that some of the antigenic determinants were altered in these strains (Goelz, 1979).

Immunoprecipitation experiments also indicated that inner membranes contained proteins that were immunogenically

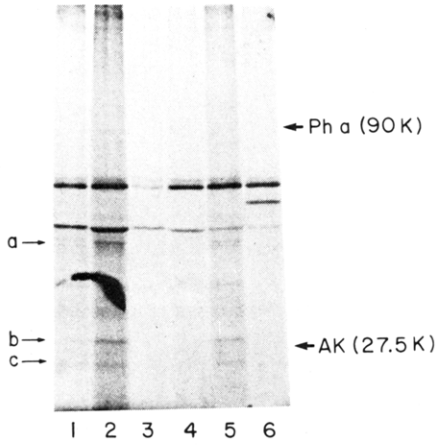

FIGURE 6: Polyacrylamide gel electrophoresis of ^{35}S-labeled inner membrane proteins precipitated by adenylate kinase antibodies. Inner membranes were prepared from strain VL1A/pVL1, solubilized in 0.1% Triton X-100, and centrifuged in a Brinkman microfuge for 15 s. To 20 μL of the supernatant (approximately 1.5×10^5 cpm/μL) was added 200 μL of Pansorbin, prepared by the method of Kessler (1975), and the solution was incubated for 1 h at 0 °C. After a 10-s centrifugation, 20 μL of this supernatant was incubated with antibody for 4 h at 0 °C. Pansorbin (50 μL) was then added to each sample. The samples were incubated for 5 min at 0 °C and centrifuged for 10 s, and the pellets were washed twice with 200 μL of NET 2 buffer (Kessler, 1975) and once with 200 μL of H_2O, resuspended in 35 μL of $NaDodSO_4$ gel sample buffer, and applied to a 10% gel. The gel was autoradiographed. The samples contained the following: lane 1, adenylate kinase antiserum (20 μg); lane 2, adenylate kinase antiserum (100 μg); lane 3, preimmune serum (200 μg); lane 4, preimmune serum (100 μg); lane 5, anti adenylate kinase IgG (75 μg); lane 6, preimmune IgG (75 μg). The molecular weight markers were phosphorylase *a* (Ph *a*) and *E. coli* adenylate kinase (AK).

related to adenylate kinase. When inner membranes from cells labeled with [^{35}S]methionine were solubilized in Triton X-100 and treated with anti adenylate kinase IgG followed by *Staphylococcus aureus* cells (Kessler, 1975), a number of proteins were specifically precipitated (Figure 6). These proteins had apparent molecular weights of approximately 25 000, 29 000, and 47 000. These proteins therefore either share antigenic determinants with adenylate kinase or form a Triton-stable complex with adenylate kinase that can be precipitated by anti adenylate kinase IgG.

Discussion

We have made several key observations on the phenotype of the *adk* mutants. First, strains carrying the *adk-31* allele of adenylate kinase retain a considerable ATP pool but cease synthesis of phospholipids. The unusually high ATP pools of *adk-31* strains at the nonpermissive temperature correlate well with the temperature stability of the adenylate kinase of these mutants. However, the acyltransferase of these strains was very thermolabile. This result indicates that acyltransferase thermolability is caused by an alteration of adenylate kinase but that the alteration does not necessarily result in a temperature-sensitive adenylate kinase. The fact that the adenylate kinase from *adk-31* strains was indeed altered is shown by the anomalous behavior of the mutant enzyme during purification. Our data therefore indicate that the inhibition of phospholipid synthesis observed after shift of *adk* strains to nonpermissive temperatures cannot be attributed to depletion of intracellular ATP pools. It should be noted that Hennen et al. (1978) have reported that phospholipid synthesis in *E. coli* is much less sensitive to decreased ATP levels than are the synthetic pathways of DNA, RNA, and protein. For example, Hennen et al. (1978) found that decreasing the intracellular ATP pool by 6-fold inhibited phospholipid syntheses by only 2-fold whereas RNA synthesis was completely inhibited. Moreover, a 2-fold decrease in the intracellular ATP concentration had no effect on the rate of phospholipid synthesis (Hennen et al., 1978). For these reasons we believe that the argument put forth first by Snider & Kennedy (1977) and subsequently by Raetz (1978) can be discarded. These workers had argued that the inhibition of phospholipid synthesis observed in *adk* mutants was the direct result of depleted ATP pools. The phenotype of the *adk-31* strains and the data of Hennen et al. (1978) contradict this explanation.

Our attempts to demonstrate an acyltransferase–adenylate kinase interaction by more direct means had some success. We were able to alleviate the thermolability of the mutant acyltransferase by the addition of a small amount of homogeneous wild-type adenylate kinase. The amount of adenylate kinase that stabilized the mutant acyltransferase comprised less than 0.1% of the total protein in the mixture. It seems most probable that the stabilization observed could be due to replacement of acyltransferase-associated mutant adenylate kinase with normal adenylate kinase. However, Cousin & Buttin (1969) have reported that addition of small amounts of purified wild-type adenylate kinase to crude extracts of an *adk* mutant resulted in a marked stabilization of the mutant kinase activity, and thus the wild-type enzyme could stabilize (rather than replace) the mutant enzyme.

The most desirable control for these experiments would have been the addition of a homogeneous mutant adenylate kinase to the mutant membranes. Unfortunately, we were unable to purify the mutant enzymes with our standard protocol. However, the behavior of the mutant enzymes upon purification suggests that the structures of these enzymes differ greatly from those of the wild-type enzyme, and thus these adenylate kinases might not have been satisfactory controls. The stabilization of the acyltransferase is not a general protein effect. Neither ovalbumin (Table I) nor bovine serum albumin (Cronan et al., 1970; Ray et al., 1970, 1976) stabilized the mutant enzymes to thermal inactivation.

We found that freezing and thawing the membranes of the *adk* mutants before use has a major effect on the thermolability of the acyltransferase activity and on our ability to relieve the thermolability by addition of exogenous adenylate kinase (Goelz, 1979). We do not understand the freeze–thaw effect, but perhaps this procedure sufficiently disrupts the membrane to allow access of exogenous proteins to the membrane sites occupied by adenylate kinase. Similar freeze–thaw procedures have recently been used for the incorporation of soluble proteins into membranes. A particularly relevant example is that of Tokuda & Konisky (1978), who showed that a freeze–thaw cycle was required to allow colicin Ia access to its binding site on *E. coli* membrane vesicles.

It should be noted that Snider & Kennedy (1977) reported that the acyltransferase of an *adk-15* strain was not thermolabile. However, these workers used assay conditions designed to stabilize the wild-type enzyme that may have also stabilized the mutant enzyme and obscured its thermolability (particularly if the membranes had not been frozen and thawed). Another problem is that these workers grew the *adk-15* strain at 30 °C. Revertant strains readily overgrow the *adk-15* strains at this temperature, and controls for reversion were not reported.

Lightner et al. (1980) have recently reported the identification of a structural gene for the acyltransferase by in vitro molecular cloning procedures. Starting from cells enriched in the acyltransferase, these workers (Larsen et al., 1980) have identified an inner membrane protein having a molecular

weight of about 83000 as a catalytic subunit of the acyltransferase. Although the detailed purification procedure has not yet been reported, it should soon be possible to directly test for interaction between this protein and adenylate kinase. However, it should be noted that recent kinetic evidence suggests that the membrane-bound acyltransferase activity has a number of different substrate and effector binding sites and hence might be composed of several proteins (Rock et al., 1981a).

The finding that a single mutation can give rise to two altered enzyme activities is unusual but not unprecedented. A particularly relevant example may be the finding that mutations in the structural gene for the transcription termination factor, ρ, cause alterations in both ρ and the membrane-bound Ca^{2+}/Mg^{2+} ATPase of *E. coli* (Das et al., 1979).

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