In Vitro Adenylylation of Lysine-Sensitive Aspartylkinase from *Escherichia coli* TIR-8†

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ABSTRACT: Incubation of lysine sensitive aspartylkinase (AK III) from *Escherichia coli* Tir-8, with either [^{3}H]ATP or [α-^{32}P]ATP, Mg^{2+} and *E. coli* extracts, results in a time-dependent incorporation of radioactive nucleotide (2–10%/subunit) into AK III. The nucleotide remains bound to the AK III after chromatography on Sephadex G-25, Sephadex G-200, DEAE-Sephadex, and polyacrylamide gel electrophoresis. Covalent attachment of the nucleotide is further demonstrated by isolation of labeled tryptic peptides by both Dowex 1-X2 chromatography and mapping of the peptides on paper by electrophoresis and chromatography. The bound nucleotide is sensitive to hydrolysis by snake venom phosphodiesterase indicating that the nucleotide is bound in a phosphodiester linkage.

The control of regulatory enzymes by enzyme-catalyzed covalent modification has recently been extensively reviewed (Holzer and Duntze, 1971). Control by phosphorylation and dephosphorylation has been observed in a number of systems (Villar-Palasi and Larner, 1961; Mendicino *et al.*, 1966; De Lange *et al.*, 1968; Linn *et al.*, 1969).

Glutamine synthetase from *Escherichia coli* has been shown to be regulated by the reversible incorporation of one AMP per subunit (Wulff *et al.*, 1967; Shapiro *et al.*, 1967; Kingdon *et al.*, 1967). The incorporation of AMP into glutamine synthetase results in a change in the enzyme's divalent metal requirement, pH optima, and response to various feedback inhibitors (Shapiro and Stadtman, 1970). Adenylylated glutamine synthetase can be reactivated by a catalytic deadenylylation (Shapiro, 1969).

Recently, both the adenylylating and deadenylylating activities have been shown to be part of a multicomponent enzyme complex (Anderson *et al.*, 1970; Hennig *et al.*, 1970). The activity of this complex appears to be regulated by a protein whose activity may be modulated by the incorporation of UMP (Brown *et al.*, 1971). The adenylylation reaction is activated by glutamine and inhibited by α-ketoglutarate; the deadenylylation reaction is activated by ATP, UTP, and α-ketoglutarate (Stadtman *et al.*, 1968).

In the previous paper (Niles and Westhead, 1973) we have shown that the lysine-sensitive aspartylkinase activity from *E. coli* K_{12} is rapidly eliminated at the end of log-phase growth. Furthermore, enzyme from different stages of growth shows different degrees of subunit association and different contents of a covalently bound 260-nm-absorbing moiety. In this paper, we will present evidence for the catalytic adenylylation of lysine-sensitive aspartylkinase from *E. coli* Tir-8, *in vitro*.

Materials

Source of Radioactive Compounds. [^{3}H]ATP (7.39 Ci/mmol) and [α-^{32}P]ATP (3.5 Ci/mmol) were obtained from New England Nuclear Corp., Boston, Mass. The [^{3}H]ATP is shipped in 50% ethanol–water solution, and a stock aqueous solution was prepared by passing a stream of nitrogen over an aliquot of the solution of 0° until the ethanol was removed (Hennig and Ginsburg, 1971). The radioactive ATP was stored at −15°.

Preparations for solubilization (Protosol) and for scintillation counting (Aquasol) were purchased from New England Nuclear Corp. Deoxyribonuclease (II), ribonuclease (II), and snake venom phosphodiesterase were obtained from Sigma Chemical Corp.

Sources of other materials used are given in the previous paper (Niles and Westhead, 1973). Buffers used were: GL buffer: 1.0 mM potassium phosphate (pH 7.0)–1.0 mM L-lysine–2 mM $MgCl_2 \cdot 6H_2O$–1 mM EDTA–0.1 mM dithiothreitol; adenylylating buffer: 40 mM Tris (Cl) (pH 8.0)–50 mM $MgCl_2 \cdot 6H_2O$–10.0 mM L-lysine–0.1 mM dithiothreitol.

The source of bacteria, culture conditions and procedures for purification of AK III[1] (designation of Cohen, 1969) are given in the previous paper (Niles and Westhead, 1973).

Methods

Preparation of the Adenylylating Extract. E. coli Tir-8 (10 g) was suspended in 30 ml of adenylylating buffer. The suspension was sonicated in an ice bath for five 1-min intervals and centrifuged at 30,000*g* for 10 min to remove the debris. To the supernatant was added enough streptomycin sulfate to bring the final concentration to 3% (w/v). The pH was adjusted to 7.6 and the sample was centrifuged for 10 min at 30,000*g*. The precipitate, containing nucleic acids, was discarded. Enough ribonuclease and deoxyribonuclease were then added to the supernatant to bring it to 20 μg/ml of each. The digestion was allowed to proceed for 4 hr at 37°. The solution was clarified by centrifugation and the supernatant was concentrated to 5 ml in an Amicon membrane concentrator. The solution (1 ml) was passed over a G-25 Sephadex column (1.1 × 20 cm), and fractions were collected. The protein-containing fractions were combined and sterilized by passage through a 0.22 μ Millipore filter.

Adenylylation Procedure. INCUBATION MIXTURE. Aliquots of aspartylkinase III, 0.4–1.3 mg in GL buffer, were incubated

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[1] Abbreviation used is: AK III, lysine-sensitive aspartylkinase.

with 0.5–1.0 mg of extract, 5.0 mM radioactive ATP, either ^{3}H (5.5×10^{6} cmp/μmol) or ^{32}P (1.2×10^{7} cpm/μmol), 100 μg of phosphoenolpyruvate, 10 μg of pyruvate kinase in adenylylating buffer (pH 8.0) in a final volume of 0.2–0.5 ml. The final mixture was pH 7.6. The incubation was carried out in a conical centrifuge tube, at 30 or 36° for a specified length of time.

Adenylylation Assays. SEPHADEX G-25 ASSAY. At various times, a 0.1- to 0.5-ml aliquot of the incubation mixture was passed over a Sephadex G-25 (fine grade) column (2.5×18 cm) previously equilibrated with adenylylating buffer. Fractions of 1 ml were assayed for AK III activity and the samples were counted in 10 ml of Aquasol. The number of nmoles of ATP associated with the enzyme was calculated for the peak samples and the average was taken.

As a control, incubation mixtures were prepared lacking AK III. At specified times, an aliquot from the control incubation was mixed with AK III and passed immediately over the G-25 Sephadex column. The AK III concentration in these controls was the same as in the parallel incubations. The average nmoles of ATP associated with the AK III was calculated as above.

FILTER PAPER DISK ASSAY. The filter paper disk assay of Mans and Novelli (1961) was used; 0.04- to 0.10-ml aliquots of the incubation mixture were removed and placed on labeled, 2.5-cm filter paper disks, pinned to a styrofoam base. A control incubation mixture was prepared lacking AK III. As an aliquot of the control was added to the disk, AK III was also added. Without allowing the incubation mixture to completely dry, the disk was dropped in a 2-l. volume of cold 10% CCl_3COOH–0.55% K_2HPO_4–1.1% $Na_2P_2O_5$. The CCl_3COOH precipitates the protein into the paper disk; the K_2HPO_4 and $Na_2P_2O_5$ facilitates the washing of the free nucleotides from the disk (P. Parsons and R. Rascati, 1971, personal communication). The washing procedure continued as discussed by Mans and Novelli (1961), omitting the hot CCl_3COOH step.

Two-Dimensional Separation of Peptides on Paper. The labeled peptides (5 mg) were suspended in 50 μl of electrophoresis buffer (pyridine–acetic acid–water, 400:16:3600) (Bennett, 1967). The suspension was centrifuged at 6000 rpm for 10 min. The supernatant was applied to an 18×18 in. paper and subjected to electrophoresis in pH 6.5 buffer at 2600 V, 70 mA for 90 min. Standards were run which contained histidine, proline, glutamic acid, lysine, and AMP.

After the electrophoresis, the paper was dried and the peptides were separated in the perpendicular direction by descending chromatography in the organic phase of a butanol–acetic acid–water solution (4:1:5) (Anfinsen *et al.*, 1959) for 15 hr. The same amino acid and AMP standard was also run.

The paper was dipped in ninhydrin (Bennett, 1967), the spots were cut out, and the radioactivity of each was counted in 10 ml of Aquasol.

Dowex 1-X2 Chromatography. The Dowex 1-X2 resin was precycled as outlined by Schroeder (1967). The resin was degassed and poured into a jacketed (0.8×70 cm) column and packed by pumping at 0.5 ml/min. The column was equilibrated with degassed pH 9.4 buffer. The temperature of the column was controlled at 38°.

Peptides were dissolved in 3 ml of pH 9.4 buffer and the pH was adjusted to pH 10 with KOH. The peptides were adsorbed to the column and eluted stepwise with 40 ml of pH 9.4 buffer, 120 ml of pH 8.4 buffer, 200 ml of pH 6.5 buffer, 240 ml of 0.5 N acetic acid, and 400 ml of 2 N acetic acid; 2-ml fractions were collected.

The peptide elution profile was determined by the method of Moore and Stein (1954). One milliliter of each fraction was counted in 10 ml of Aquasol.

Disc Electrophoresis. Enzyme (100-μg samples) was electrophoresed at 5.0 mA/tube in pH 8.3 buffer according to the method of Ornstein and Davis (1964). The gels were sliced into 2.5-mm sections, dissolved by incubation in 0.1 ml of 30% H_2O_2 at 50°, and counted in 10 ml of Aquasol. Gel slices from a duplicate gel were suspended in 0.5 ml of GL buffer and crushed with a stirring rod. The extracts were assayed for AK III activity. A third gel was stained for 1 hr with 0.1% Amido Black in 7% acetic acid and destained electrolytically.

Phosphodiesterase Treatment. Enzyme-labeled [α-^{32}P]ATP was repurified by chromatography on Sephadex G-25 (fine), DEAE-Sephadex, and Bio-Gel HT (Niles and Westhead, 1973). The active enzyme from the last column was concentrated by ultrafiltration to 1 ml. The sample was taken up in 10 ml of adenylylating buffer, reconcentrated to 1 ml, and sterilized by passage through a Millipore filter (0.22 μ). The sample contained 0.42 mg of AK III, 6000 cpm of ^{32}P.

To 1 ml of the sample was added 10 μg of snake venom phosphodiesterase and it was incubated at 37°. At various times, a 100-μl aliquot was removed and to it was added 20 μl of 70% perchloric acid. After 30 min at 0°, the precipitate was collected and dissolved in 0.1 ml of Protosol. The sample was counted in 10 ml of Aquasol.

Results

From the previous work reported in Niles and Westhead (1973), it appears that AK III is under a form of control different from induction–repression, or feedback inhibition. It was found that the enzyme activity peaks at the end of log-phase growth and that the enzyme can be isolated in two forms differing in quaternary structure and uv absorption spectra.

In several purifications of AK III from various stages of exponential growth, the uv spectrum revealed that there were variable amounts of a 260-nm-absorbing moiety incorporated into AK III. Assuming that this moiety was AMP and that the $A_{280}:A_{260}$ ratio of unmodified protein was about 2.0, it could be calculated that from 0.2 to 0.9 mol of AMP was incorporated per mol of AK III subunit in various preparations. It was noted that the increase in the level of 260-nm-absorbing moiety was generally coincident with the decrease in AK III activity at the beginning of stationary-phase growth.

Covalent Attachment in Vivo of 260-nm-Absorbing Moiety. The AK III sample used for the subunit molecular weight determination in 6 M guanidine hydrochloride (Niles and Westhead, 1973) exhibited a very high 260-nm absorbance in the native state. This protein, denatured in 6 M guanidine hydrochloride, was passed over Sephadex G-25 preequilibrated with 6 M guanidine hydrochloride and 1 mM bromoacetic acid to remove the mercaptoethanol and prevent reoxidation of the sulfhydryl groups of the protein. An absorption spectrum of this denatured enzyme showed that the 260-nm-absorbing material remained bound to the denatured enzyme. Therefore, it appears that the 260-nm-absorbing material is not bound to AK III by noncovalent adherence to a high-affinity site on the enzyme.

The variable uv spectrum and the peak in AK III activity at the beginning of the stationary-phase growth suggested the possibility that AK III might be adenylylated in a manner similar to glutamine synthetase. To test this hypothesis, the *in vitro* incorporation of ATP into AK III was attempted.

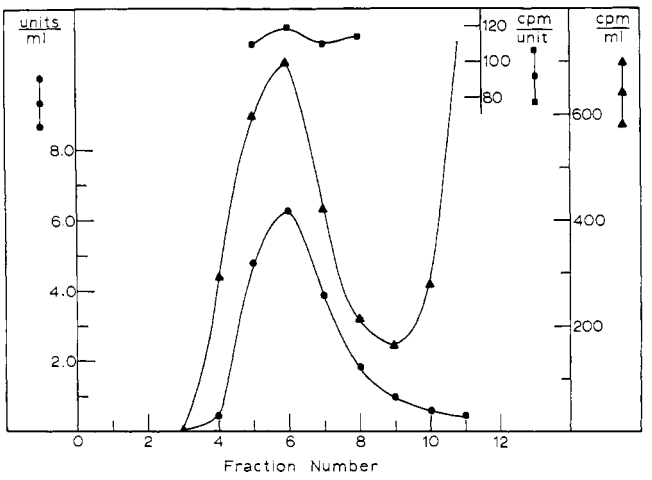

FIGURE 1: Sephadex G-25: a standard incubation mixture containing 5 mM [^{3}H]ATP (5.5×10^6 cpm/μmol), 0.5 mg AK III and 0.5 mg of extract protein in 0.2 ml, was passed over a G-25 Sephadex column (2.5×18 cm) and 1.0-ml fractions were collected. (●) AK III; (▲) cpm of ^{3}H; (■) cpm/unit of activity.

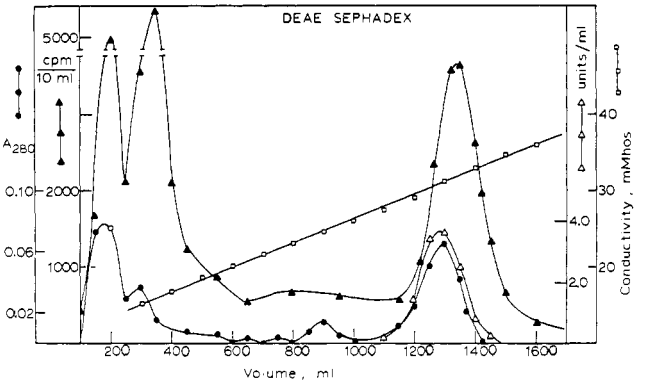

FIGURE 2: DEAE-Sephadex: 35 mg of AK III, 5.7 mM [α-^{32}P]ATP (1.2×10^7 cpm/μmol), and 20 mg of extract were incubated for 23 hr at 36°. The protein was precipitated with 50% saturated ammonium sulfate and the precipitate dialyzed into DEAE (low salt) buffer by passage over a G-25 Sephadex Column (4.5×20 cm). The protein peak was adsorbed to a DEAE-Sephadex column (2.5×10 cm) and eluted with a linear gradient made from 1.5 l. of DEAE (low salt) and 1.5 l. of DEAE (high salt) buffer; 10-ml fractions were collected. (▲) cpm; (Δ) AK III; (●) A_{280}; (■) conductivity.

In Vitro Attachment of Radioactive ATP

Sephadex G-25. As a preliminary test of incorporation a sample of purified AK III was incubated with a crude extract of *E. coli* containing ^{3}H-labeled ATP. As described in Methods the incubation mixture (0.2 ml) was passed over a Sephadex G-25 fine column and 1-ml fractions were collected. AK III activity and the radioactivity were measured in each tube. It can be seen from Figure 1 that the specific activity (cpm/unit) is constant over the area of the protein peak. Soon after the peak is eluted, the unbound radioactivity comes out, adequately separated from the protein.

In order to prove that the counts which eluted were covalently bound and did not just cochromatograph with protein because of a high-affinity constant, another experiment was carried out in which both the control column and the sample column were equilibrated with 5 mM unlabeled ATP and the incubation mixture was eluted with 5 mM unlabeled ATP. There was no significant decrease in the background or in the level of incorporation into the AK III or control samples indicating that most of the counts associated with the protein fractions are unable to be exchanged with the free ATP in the column and may therefore be covalently bound to the protein.

In several preliminary experiments, in order to test further the specificity of incorporation of a form of ATP into AK III, the eluant from the G-25 column was further chromatographed on DEAE Sephadex or Sephadex G-200, or fractionated by electrophoresis on gels. In each case it was found that most of the radioactivity remained with the AK III. ATP labeled with tritium or with ^{32}P in the α position behaves the same way.

In the data which follow, a single sample was purified sequentially through different procedures, using [α-^{32}P]ATP as radioactive marker. A 35-mg sample of AK III with a 280 nm:260 nm absorption ratio of 1.56 was incubated with 20 mg of extract, 20 mg of phosphenolpyruvate, 1 mg of pyruvate kinase, and 5.7 mM [^{32}P]ATP (1.2×10^7 cpm/μmol) in a final volume of 17.5 ml. After 23 hr at 36°, the reaction was ended with the addition of 17.5 ml of cold GL buffer, saturated with ammonium sulfate. After 30 min the sample was centrifuged at 15,000 rpm and the supernatant was discarded.

DEAE-Sephadex. The precipitate was dissolved in 5 ml of DEAE-Sephadex buffer (low salt; see Niles and Westhead, 1973) and the protein was exchanged into the same buffer by passage through a Sephadex G-25 column (4.5×20 cm).

The protein was then adsorbed to a DEAE-Sephadex column (2.5×10 cm) and eluted with a linear gradient of 1.5 l. (low salt) and 1.5 l. (high salt) of DEAE-Sephadex buffer. The enzyme was collected in 10-ml fractions, the enzyme activity and A_{280} were measured and the radioactivity was determined by the Cerenkov radiation (100% gain, window 50–1000) (Haviland and Bieber, 1970). It can be seen in Figure 2 that a large fraction of the total counts cochromatograph with the AK III activity. The early peak of radioactivity was determined to be nucleotide by its uv spectrum.

Sephadex G-200. The enzyme fractions with highest specific activity were combined and concentrated to 10 ml in an Amicon ultrafiltration device with a UM-10 membrane. The solution was passed over a G-200 column (100×2.5 cm) and eluted with GL buffer. The enzyme was assayed for both AK III activity and ^{32}P by Cerenkov radiation as before. Fractions with constant specific activity were combined and concentrated to 2.5 ml (5.7 mg/ml, 92,000 cpm).

Figure 3 is an elution profile of the enzyme from the Sephadex G-200 column. It is apparent that essentially all of the counts that were put on the column eluted with the AK III activity. The specific enzyme activity of the AK III was constant after both the DEAE-Sephadex and the Sephadex G-200 columns indicating that the enzyme is at least 90% pure. The specific radioactivity was also constant at about 3.0 mol of nucleotide/100 mol of subunit.

Gel Electrophoresis. As a further test for the adenylylation, a sample of the pooled G-200 Sephadex column fraction was electrophoresed in acrylamide gel at pH 8.3. The results are presented in Figure 4. The enzyme is essentially homogeneous and the radioactivity is associated almost exclusively with the AK III band. There is, however, a small amount of radioactivity migrating with the tracking dye. This may be due to spontaneous hydrolysis of the diester bond at pH 8.3 because there is no evidence for carry-over of nucleotide from the G-200 column (Figure 3). There is about 2.8 mol of nucleotide/100 mol of subunit.

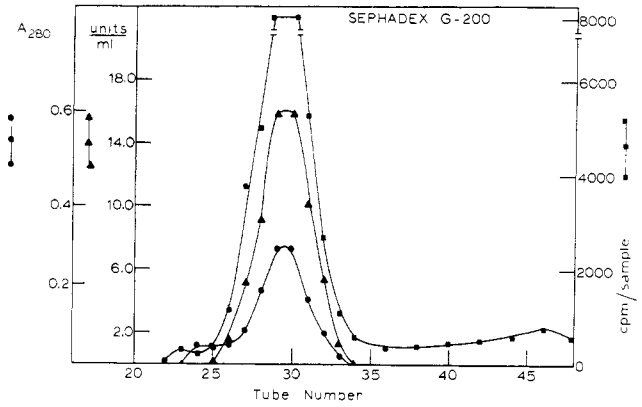

FIGURE 3: Sephadex G-200: the pooled peak (18.3 mg of AK III, 12,000 cpm) from the DEAE-Sephadex column was passed over a Sephadex G-200 column (2.5 × 100 cm); 10-ml fractions were collected. (●) A_{280}; (▲) AK III activity; (■) cpm of ^{32}P.

Kinetics Studies. A simple study of the kinetics of the incorporation was undertaken to show the enzymatic nature of the incorporation. Two assays were used to study the rate of incorporation of ATP into AK III. The first used was the G-25 Sephadex column separation of the unbound radioactivity from the protein fraction, and second, the filter paper technique (Mans and Novelli, 1961).

Time Course. Since it is possible that there would be both unbound ATP, and ATP bound to another protein such as glutamine synthetase in the extract, a control column was used. To this column was added an aliquot of an incubation mixture which had no AK III in it. AK III was added when the incubation mixture was put on the column. In this way, there would be no incorporation into AK III and this blank would compensate for any nonspecific binding of ATP to AK III and any incorporation into a protein from the crude extract.

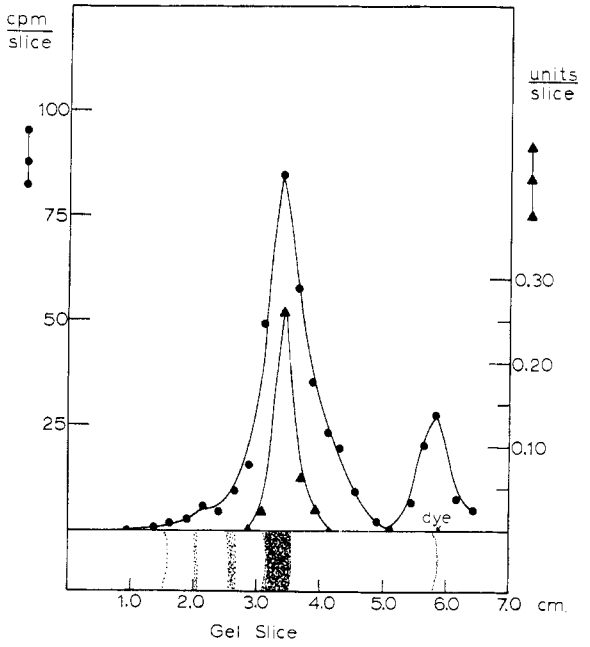

FIGURE 4: Disc gel electrophoresis: discontinuous electrophoresis of 100-μg aliquots of AK III eluted from Sephadex G-200 (Figure 3) was carried out at pH 8.3. One gel was stained in 0.1% Amido Black in 7% acetic acid and destained electrolytically. Two gels were sliced into 2.5-mm sections. The slices from one gel were dissolved in 0.1 ml of 30% H_2O_2 at 50° and the ^{32}P was counted in 10 ml of Aquasol. The slices from the other gel were suspended in 0.5 ml of GL buffer and the AK III activity was measured. (▲) cpm/slice of ^{32}P; (●) units of AK III/slice.

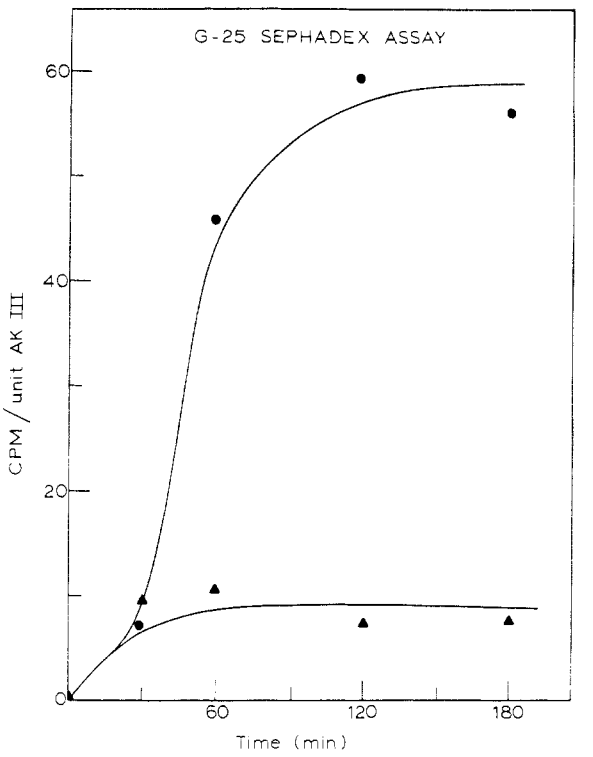

FIGURE 5: Time course of adenylylation: two incubations, both containing 5 mM [^{3}H]ATP (5.4 × 10^6 cpm/μmol) and 1.0 mg of extract in 0.5 ml, were set up. One incubation contained 1.0 mg of AK III, the other lacked AK III. At various times, 100-μl aliquots were removed from each. The AK III incubation was passed over a Sephadex G-25 (fine) column and the elution was monitored. AK III (0.2 mg) was added to each 100-μl aliquot of the control incubation at the Sephadex G-25 column. The amount of [^{3}H]ATP incorporated into each sample was calculated at various time points. (●) AK III incubation; (▲) control incubation.

This assay procedure was carried out after varying time intervals and the amount of radioactivity bound to high molecular weight material in either the AK III incubation or the control was determined. The zero-time point for both the AK III incubation and the control were subtracted from subsequent points. The results are presented in Figure 5. It can be seen that there is an increase in incorporation into the AK III sample which levels off after about 2 hr. The control sample also increases with time, but only to a level of about 15% of that of the AK III incubation. This is consistent with the idea that there is specific incorporation of ATP into AK III, to a level of about 2% of the maximum expected in this experiment. The zero-time point was 25 cpm/unit of enzyme activity.

The time course of the adenylylation of AK III was also examined by the filter paper disk assay and similar results were found. The extent of incorporation varied from 2 to 10% of the maximum expected, with different preparations of adenylylating extract. It should be pointed out that the A_{280}:A_{260} ratio of the AK III used in these studies was between 1.56 and 1.7, indicating that the enzyme already contained from 0.25 to 0.5 mol of nucleotide per subunit at zero time.

ATP Dependence. If this is true adenylylation of AK III,

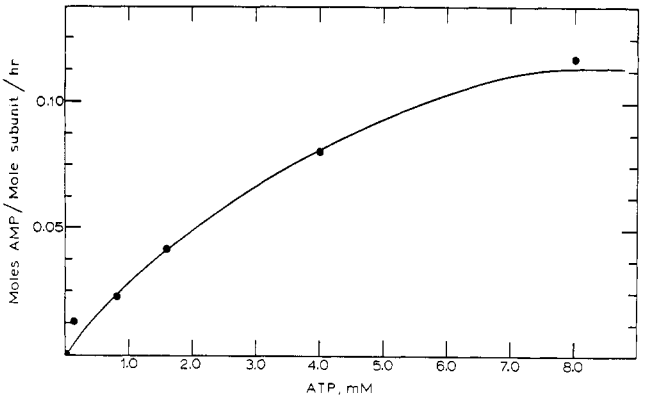

FIGURE 6: ATP concentration dependence: a series of incubations were set up as in Figure 5 with ATP varying from 1 μM to 5 mM. The incubations were assayed by the G-25 Sephadex method after 60 min at 37°.

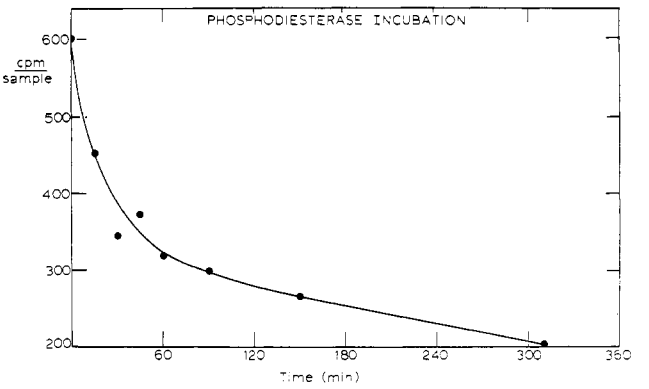

FIGURE 7: Phosphodiesterase removal of label: 0.42 mg of AK III labeled with 6000 cpm of [α-^{32}P]ATP was incubated with 10 g of snake venom phosphodiesterase. At various times, 100 μl was removed and the protein was precipitated with 12% perchloric acid. The precipitate was counted in Aquasol (NEN). Details of the incubation are given in Methods.

then the adenylylation of AK III must be dependent on the molar concentration of ATP in the incubation. The amount of radioactive ATP in the incubation was kept constant and the amount of cold ATP was varied to give different concentrations of ATP. The degree of incorporation of ATP into the enzyme was measured by the G-25 Sephadex column assay and the results are presented in Figure 6. As would be expected, the rate of incorporation of ATP into AK III is dependent on the concentration of ATP in the incubation. The apparent "K_m" for ATP from these data (about 3.0 mM) cannot be considered a true K_m, since both this parameter and the maximum incorporation of 3% may be partly determined by ATPases in the extract or diesterases which cleave the nucleotide from the AK III.

To test further the enzymatic nature of the incorporation, both AK III and the extract concentration dependences were examined. It was found that the rate of incorporation was dependent upon the concentration of extract in the incubation. Furthermore, both the extent of incorporation and the initial rate of incorporation were dependent upon the concentration of AK III in the incubation mixture.

Phosphodiesterase Sensitivity. In the case of glutamine synthetase (Shapiro *et al.*, 1967), the bound nucleotide was shown to be cleaved by snake venom phosphodiesterase indicating that the AMP is bound in a phosphate diester linkage. Details of a similar experiment with radioactive ATP-labeled AK III are given in Methods. The results of the experiment are shown in Figure 7. The radioactivity remaining in the CCl_3COOH precipitate is shown to decrease with time, eventually approaching complete removal of the ATP. These results indicate the phosphodiester nature of the aspartylkinase–nucleotide bond as well as providing further evidence for the covalent nature of the association.

Radioactive Peptide Isolation. To further establish the covalent nature of the bond between the enzyme and nucleotide and to prepare the way for identification of the site of attachment, we have carried out preliminary separations of tryptic peptides from the labeled enzyme. For this purpose, we used the labeled enzyme repurified through Sephadex G-200 (Figure 3). Of the 2.5 ml of concentrated enzyme, a small amount was used for gel electrophoresis (Figure 4) and the rest for tryptic digestion.

To 2.45 ml of enzyme was mixed 8 ml of 0.12 M NH_4HCO_3–10 M urea–0.1 M mercaptoethanol (pH 8.2). After 4 hr at 45°, the solution was made 0.5 M in iodoacetamide and the pH was adjusted to 8.6 with concentrated NH_4OH. After 15 min, the solution was made 5.0 M in mercaptoethanol and dialyzed against 5 l. of H_2O at 4° with changes every 12 hr for 2 days.

The protein solution was adjusted to pH 8.4 with the addition of 0.01 M NH_4OH. Twenty microliters of 0.02% Phenol Red was added as a pH indicator; 0.1 ml of a 2-mg/ml solution of Tos-PheCH_2Cl-treated trypsin was added to the protein at 37° and the hydrolysis was monitored by the dropwise addition of 0.02 M NH_4OH to maintain the color of the dye. After the hydrolysis was complete, the peptides were lyophilized.

In developing tryptic mapping procedures for AK III peptides (Niles, 1972), it was found that the peptides were not completely soluble in any buffer tested. When a 5-mg sample of the peptides was suspended in pH 6.5 buffer, it was found that greater than 50% of the radioactivity was in the supernatant. This soluble sample was subjected to electrophoresis in pH 6.5 buffer followed by chromatography as described in Methods. Ninhydrin-stained spots were cut out and the radioactivity was measured. AMP was added to the standard amino acids to follow its migration. The results are shown in Figure 8. There were four spots of radioactivity. Spot 1 is the origin and probably corresponds to partially digested protein; it had about 75 cpm. Spots 2 and 3 did not migrate under electrophoresis but did under chromatography; they had 55 and 75 cpm, respectively. Spot 4, which migrated in both dimensions, contained the largest amount of radioactivity, 105 cpm. The total level of radioactivity corresponds to about 20% of the radioactivity applied, assuming no quenching of the radioactivity by the paper or the ninhydrin stain.

An alternative procedure for separating the peptides was carried out by passage of 18 mg of labeled peptides over a Dowex 1-X2 column (Schroeder, 1967). The elution profile is shown in Figure 9. It can be seen that there is only one peak of radioactivity which corresponds to about 15% of the radioactivity put onto the column. This peak of radioactivity corresponds to a ninhydrin peak, indicating the presence of a peptide. No determination of the elution behavior of AMP was carried out on the column. The ninhydrin peak and the radioactivity peak both have a shoulder indicating inhomogeneity of the peak. This is consistent with the fact that there are at least three radioactive spots found in the pH 6.5 tryptic map.

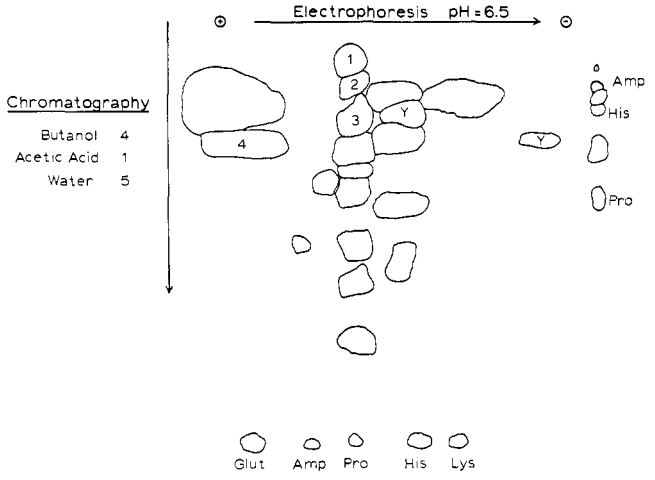

FIGURE 8: Tryptic mapping of the adenylylated peptides: the soluble portion of 5 mg of AK III peptides (as described in text) was electrophoresed at 2600 V for 90 min in pH 6.5 buffer. The paper was chromatographed for 15 hr in the organic phase of a butanol–acetic acid–H_2O (4:1:5) solvent for 15 hr at 24°. The peptides were developed by ninhydrin stain and the ^{32}P was measured in each spot by suspending the cut-out spots in 10 ml of Aquasol.

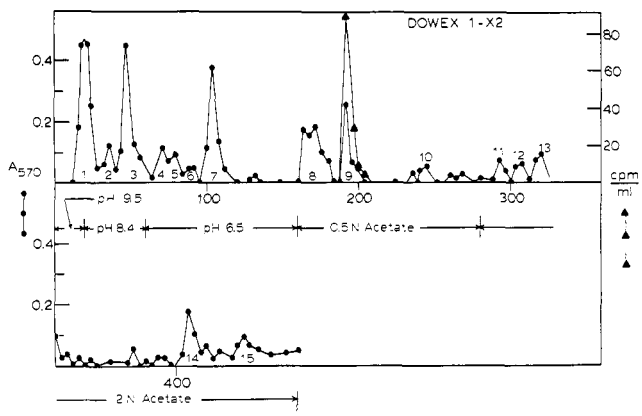

FIGURE 9: Dowex 1-X2 chromatography of the adenylylated peptide: 10 mg of AK III peptides (1.5×10^4 cpm) were dissolved in pH 9.4 buffer and adsorbed to a Dowex 1-X2 column (0.8×70 cm). The peptides were stepwise eluted with 40 ml of pH 9.4, 80 ml of pH 8.4, 200 ml of pH 6.5 buffer, 240 ml of 0.5 N acetate, and 400 ml of 2 N acetate; 2.0-ml fractions were collected; 1.0-ml of a fraction was used for the ninhydrin reaction and 1.0 ml was counted in Aquasol (NEN). (●) A_{570}; (▲) cpm of ^{32}P.

A large majority of the counts did not elute from the column even at 1.0 N HCl. It is not known what the nature of the counts might be.

Discussion

The behavior of AK III activity during growth and the isolation of two forms of the enzyme, one with a low 260-nm absorbance during the most active stage and a high 260-nm absorbance at the least active stage (Niles and Westhead, 1973), brought to mind a similarity with glutamine synthetase from *E. coli W* (Holzer, 1969). Both enzymes catalyze analogous reactions: glutamine synthetase phosphorylates glutamic acid and subsequently adds NH_3 to form glutamine. Aspartokinase phosphorylates aspartic acid and is activated by NH_4^+. Both enzymes have a subunit molecular weight of 48,000. Both are feedback inhibited by a variety of metabolic end products. The substrates for both enzymes are derived primarily from the Krebs cycle intermediates, α-ketoglutaric acid or fumarate and oxaloacetic acid. Aspartate and glutamate are intimately involved in NH_3 metabolism not only through the transamination of oxaloacetic acid and α-ketoglutarate, but also in the formation of asparagine and glutamine. Finally, both AK III and glutamine synthetase show a variable 260-nm absorption and the increase in the 260-nm-absorbing species is coincident with a decrease in the concentration of active enzyme (Wulff *et al.*, 1967; Kingdon *et al.*, 1967; Shapiro *et al.*, 1967). For these reasons, this study of the possible adenylylation of AK III was undertaken.

The results demonstrate that low levels of nucleotide, 2–10%/subunit, are covalently bound to AK III by an enzyme in the crude extract of *E. coli* Tir-8. The nucleotide bound has been shown to contain at least the α-phosphate and the adenosine portion of ATP by use of [^{3}H]ATP and [α-^{32}P]ATP. The nucleotide is covalently bound to AK III and not to an impurity, since the nucleotide remains bound to AK III after chromatography on Sephadex G-25 (Figure 1), DEAE-Sephadex (Figure 2), Sephadex G-200 (Figure 3), and disk electrophoresis (Figure 4). It is important to note that glutamine synthetase or RNA polymerase, previously shown to be adenylylated (Holzer and Duntze, 1971), would be separated from AK III on G-200 Sephadex since their molecular weights are much greater than that of AK III. The nucleotide is released by incubation of the labeled enzyme with snake venom phosphodiesterase (Figure 7), indicating that the nucleotide is bound in a phosphate ester with the enzyme.

In contrast to the stable tyrosine–AMP diester in glutamine synthetase (Shapiro and Stadtman, 1968a,b), the nucleotide in AK III appears to be fairly readily hydrolyzed. During disc electrophoresis at pH 8.3 (Figure 4), about 10% of the bound nucleotide was hydrolyzed. Recoveries of only 15% were achieved when the labeled peptides were either chromatographed on Dowex 1-X2 (Figure 9), or were separated in two dimensions on paper by electrophoresis at pH 6.5 and chromatography in a butanol–acetic acid–water solvent (Figure 8).

Tryptic digestion of the labeled enzyme revealed at least three radioactive species by two-dimensional mapping on paper. This might be accounted for by the position of the nucleotide on the polypeptide chain, affecting the ease of digestibility of the polypeptide resulting in several species of polypeptide. Chromatography of the AK III peptides on a Dowex 1-X2 resin revealed only a single ninhydrin-positive peak which contained radioactivity (Figure 9), but the peak exhibits a shoulder indicating either the presence of at least one closely related radioactive polypeptide, or continuing breakdown of the peptide.

Taking into account the apparent susceptibility of the AMP ester to hydrolysis during the disc electrophoresis (Figure 4), it is not surprising that the counts were not recovered quantitatively either from the acidic tryptic maps or from the Dowex column which goes from basic to acidic extremes. Other procedures for separation of these peptides will have to be developed before attempts are made to analyze the labeled peptide.

The low level of *in vitro* incorporation of the nucleotide precluded a study of the effects of *in vitro* adenylylation on the enzyme. This low level may be explained on the basis of an unstable adenylylating enzyme present in low concentrations in the extracts, our lack of knowledge of positive and negative effectors of the AK III adenylylating enzyme and the possible

presence of an enzyme which hydrolyzes the substrate or the product. It is known that glutamine synthetase adenylyltransferase is only 5% active in the absence of glutamine (Kingdon *et al.*, 1967) and is inhibited by such compounds as phosphoenolpyruvate, fructose diphosphate, α-ketoglutarate, ATP, and UTP (Ebner *et al.*, 1970). It is not surprising, then, that at this stage, the level of AMP incorporation is low, since we are working with a crude *in vitro* system and do not know the optimum conditions for the adenylylation of AK III.

From the uv spectrum of a sample of AK III isolated from late-stationary-phase bacteria (Niles and Westhead, 1973), it could be calculated that there is about 1 mol of nucleotide bound per mol of AK III subunit. Since this 260-nm-absorbing species remains bound to the enzyme denatured in guanidine hydrochloride and mercaptoethanol, it is apparently covalently bound. We find it reasonable, therefore, to assume that while the level of adenylylation that is observed *in vitro* is low, it is actually a manifestation of an *in vivo* process.

The nature of the bound 260-nm-absorbing moiety remains to be ascertained. Although ATP seems as a substrate *in vitro* it is possible that another nucleotide derivative is the donor *in vivo*. It is also possible that AK III is serving as a poor substrate, *in vitro* for the glutamine synthetase adenylyltransferase. The isolation of AK III from stationary-phase cells, with a uv spectrum indicating 0.9 mol of nucleotide/mol of subunit, argues against the possibility of an *in vitro* artifact.

Determination of the regulatory significance of the apparent adenylylation awaits further experimentation.

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