Glutamine Synthetase Adenylyltransferase from *Escherichia coli:* Purification and Physical and Chemical Properties†

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ABSTRACT: The glutamine synthetase adenylyltransferase (EC 2.7.7.42), which catalyzes the adenylylation and deadenylylation of glutamine synthetase in *E. coli,* has been stabilized and purified 2200-fold to apparent homogeneity. Sedimentation and electrophoresis studies show that the native enzyme is a *single* polypeptide chain of 115 000 ± 5000 molecular weight with an isoelectric pH (pI) of 4.98, a sedimentation coefficient ($s_{20,w}^0$) of 5.6S, and a molar frictional coefficient (f/f_0) of 1.52. An α-helical content of ~25% and ~28% β-pleated sheet and ~47% random coil structures were estimated from circular dichroism measurements. The amino acid composition of the protein has been determined. The intrinsic tryptophanyl residue fluorescence of adenylyltransferase is twofold greater than that of L-tryptophan; this property has been used to monitor ligand-induced conformational changes in the enzyme. Activators of the adenylylation reaction (ATP, L-glutamine, or the *E. coli* P_{II} regulatory protein) produced an enhancement of fluorescence; α-ketoglutarate, an inhibitor of adenylylation and an activator of deadenylylation, caused a net decrease in fluorescence. The adenylyltransferase has separate interaction sites for L-glutamine and the regulatory P_{II} protein.

Glutamine synthetase activity in *Escherichia coli* is regulated by adenylylation and deadenylylation, reactions 1 and 2, respectively (see reviews of Holzer, 1969; Stadtman and Ginsburg, 1974).

$$\text{GS} + \text{ATP} \xrightarrow[\text{Me}^{2+}]{\text{ATase-[P}_{\text{IIA}}\text{]}} \text{AMP-GS} + \text{PP}_i \qquad (1)$$

$$\text{AMP-GS} + \text{P}_i \xrightarrow[\text{Me}^{2+}]{\text{ATase-[P}_{\text{IID(UMP)}}\text{]}} \text{GS} + \text{ADP} \qquad (2)$$

In reaction 1, the adenylyltransferase (ATase[1]) requires ATP, Mg^{2+} (or Mn^{2+}), and unadenylylated glutamine synthetase (GS) in order to attach 5′-adenylic acid to glutamine synthetase. The catalysis is stimulated by the unmodified form of the regulatory P_{II} protein (Brown et al., 1971) and by L-glutamine (Wulff et al., 1967; Kingdon et al., 1967). The site of adenylylation in glutamine synthetase is a specific tyrosyl residue within each polypeptide chain of the native dodecamer of 600 000 molecular weight; a very stable 5′-adenylyl-*O*-tyrosyl derivative is formed upon adenylylation (Shapiro and Stadtman, 1968). Adenylylation of glutamine synthetase inactivates this enzyme in catalyzing the Mg^{2+}-dependent biosynthetic reaction in which L-glutamine is synthesized from ATP, L-glutamate, and ammonia (Mecke et al., 1966; Kingdon et al., 1967). Complete inactivation occurs when all 12 subunits of glutamine synthetase are adenylylated (Kingdon et al., 1967). In reaction 2, the adenylyltransferase combines with the uridylylated form of the regulatory P_{II} protein (Brown et al., 1971; Mangum et al., 1973; Adler et al., 1975) to catalyze a phosphorolytic cleavage of AMP from adenylylated glutamine synthetase to form ADP and unadenylylated glutamine synthetase (Anderson and Stadtman, 1970). The deadenylylation reaction is stimulated by α-ketoglutarate and ATP and requires inorganic phosphate and either Mg^{2+} or Mn^{2+} (Shapiro, 1969). The uridylylation–deuridylylation (P_{II} protein interconversions) and the adenylylation–deadenylylation reactions are the bases of two oppositely directed cascade controls of glutamine synthetase activity in *E. coli* (Stadtman and Ginsburg, 1974). Since specific metabolites have multiple targets, allosteric effects are amplified.

Some properties of the adenylyltransferase from *E. coli* B have been described (Ebner et al., 1970). However, the instability of the isolated enzyme (Wolf et al., 1972) made the physical characterization of this protein difficult. In a previous study in this laboratory (Hennig and Ginsburg, 1971), a low molecular weight form of the adenylyltransferase was isolated from *E. coli* W and partially characterized. From these and other preliminary results (Anderson et al., 1970; Hennig et al., 1970), it appeared that the adenylyltransferase possibly is composed of an active subunit and a subunit responsible for interaction with the regulatory P_{II} protein. The present investigation was undertaken in order to purify a stable enzyme conformation and to determine the subunit composition and some molecular properties of the adenylyltransferase.

Experimental Section

Materials. Uniformly labeled [^{14}C]ATP (~400 mCi/mmol) was obtained from New England Nuclear Corp. Ultrapure guanidine hydrochloride, urea, ammonium sulfate,

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[1] Abbreviations used: GS_n, glutamine synthetase with an average of *n* molar equiv of 5′-AMP groups per mole of GS (600 000*g*); ATase or adenylyltransferase, glutamine synthetase adenylyltransferase; P_{II} protein, *E. coli* regulatory protein, existing in either the unmodified form (P_{IIA}) that stimulates adenylylation or the uridylylated form (P_{IID}) that stimulates deadenylylation; PMSF, phenylmethylsulfonyl fluoride; PMPS, *p*-chloromercuriphenylsulfonate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Bis-tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)-methane; TEMED, *N,N,N′N″*-tetramethylenediamine; Bisacrylamide, *N,N′*-methylenebisacrylamide.

and sodium dodecyl sulfate, and myoglobin, bovine hemoglobin, ovalbumin, and Coomassie blue were from Schwarz/Mann. Eastman was the supplier of imidazole, dimethyl suberimidate dihydrochloride, acrylamide, and iodoacetic acid. ATP, L-glutamine, Tris base, Tes,[1] Bis-Tris, rabbit muscle enolase and phosphorylase *a*, β-mercaptoethanol, PMPS, and fluorimetric grade III imidazole were obtained from Sigma Chemical Corporation. Other special reagents were: 2-methylimidazole and triethanolamine (Aldrich); trypsin (Worthington Biochemical Corp.); bisacrylamide and TEMED (Canalco Industrial Corp.); 2,4-dimethylimidazole (Gallard-Schlessinger); bovine serum albumin, 5-times recrystallized (Pentex); rabbit muscle aldolase (Ginsburg and Mehler, 1966); streptomycin sulfate (Nutritional Biochemicals); $K_2MgEDTA$ (E-M Reagents Divisions; Brinkmann Instruments); PMSF was from Calbiochem and stored as a stock 0.1 M solution in 70% 2-propanol; DEAE-cellulose (Whatman DE-52) was treated with acid and base, degased, defined, and equilibrated with buffer before use. AH-Sepharose 4B (Pharmacia) and Agarose 1.5m, 200–400 mesh (Bio-Rad), were stored in 0.01% sodium azide. All other special chemicals were as reported previously (Hennig and Ginsburg, 1971).

Glutamine synthetase preparations (containing an average of 1 mol equiv of 5′-AMP) were purified from *E. coli* W grown according to Phares (1971) by the procedure of Woolfolk et al. (1966).[2] Assays and enzyme concentration determinations were as described by Shapiro and Stadtman (1970). Stock GS_1 solutions (14–16 mg/ml) for adenylyltransferase assays were prepared in 10 mM imidazole–10 mM $MgCl_2$ buffer at pH 7.4 (Hennig and Ginsburg, 1971).

The P_{II} regulatory protein (a gift from Dr. S. P. Adler) had been purified to electrophoretic homogeneity from *E. coli* W (Adler et al., 1974, 1976) in predominantly the unmodified form that stimulates adenylylation of glutamine synthetase (Mangum et al., 1973; Stadtman and Ginsburg, 1974).

Adenylyltransferase Assays. Two adenylylation assays were used.

(1) Radioactive Assay. The incorporation of [^{14}C]AMP into unadenylylated glutamine synthetase was measured by trichloroacetic acid precipitable radioactivity as previously described (Hennig and Ginsburg, 1971).[3] Adenylyltransferase fractions were diluted into 50 mM Tris-HCl (pH 8) to maintain the pH at pH ≥7.8. Assays contained a saturating concentration of unadenylylated glutamine synthetase (tested for each stock GS solution). Throughout, 1 unit of adenylyltransferase is defined as the amount of enzyme catalyzing the incorporation of 1 nmol of [^{14}C]AMP into GS (215–300 μg) per min at 37 °C.

(2) Colorimetric Assay. A two-step colorimetric assay was developed consisting of (a) incubation with unadenylylated GS under adenylylating assay conditions followed by (b) an assay of the remaining unadenylylated GS, using the γ-glutamyl transferase assay of GS at pH 7.15 with Mg^{2+} plus Mn^{2+} under the conditions of Stadtman et al. (1970). A decrease in A_{540nm} compared with a control incubation without adenylyltransferase indicates the presence of adenylyltransferase activity in the fraction tested. (a) To a 12 × 75 mm tube were added 40 μl of a freshly prepared mixture of 6.25 mM ATP, 25 mM Tris-HCl (pH 8), 37.5 mM $MgCl_2$, 12.5 mM L-glutamine, and 8–10 μg of unadenylylated GS (sufficient GS_0 to give $A_{540nm} \simeq 1.0$ in step b); the mixture was centrifuged for 1 min at 2600 rpm to collect the small volume at the bottom of the tube. Ten microliters of the adenylyltransferase fraction[4] (diluted with cold 50 mM Tris-HCl, pH 8) was added and this mixture was incubated at 37 °C. (Included was a control tube without added adenylyltransferase, and if necessary, a control tube without GS and ATP.)[4] (b) After 15 min, 0.45 ml of GS assay mixture was added (warmed to 37 °C; prepared as described below), the mixture was incubated another 15 min at 37 °C, and 2 ml of $FeCl_3$–trichloroacetic acid solution was added to stop the reaction and develop the color (Shapiro and Stadtman, 1970); this was mixed and read at 540 nm. The observed A_{540nm} was substrated from that of a control tube to measure adenylyltransferase activity. The assay was linear to $\Delta A_{540nm} \simeq 0.20$ and it was useful for rapidly screening fractions for adenylyltransferase activity during purification and electrophoretic procedures. In addition, GS and radioactive ATP was conserved with no loss in sensitivity.

Preparation of the GS_0 Assay Mixture for Use in Step b above. For a final volume of 45 ml (pH 7.15), the following were mixed together: 7.5 ml of buffer containing 0.33 M imidazole, 0.33 M 2-methylimidazole, and 0.33 M 2,4-dimethylimidazole at pH 7.15; 1.0 ml of 0.02 M ADP (stored frozen at pH 7 as the sodium salt); 146 mg of L-glutamine, 1.0 ml of freshly neutralized 1 M NH_2OH (0.5 ml of 2 M NH_2OH·HCl + 0.5 ml of 2 M NaOH); 1.0 ml of 1 M potassium arsenate; and 25 ml of H_2O. The pH was adjusted to 7.25 with KOH or HCl and then 3.0 ml of 1.0 M $MgCl_2$ was added. The mixture was diluted to 43.5 ml while readjusting the pH to 7.15 with HCl. (This mixture may be stored at 4 °C for at least 1 week.) Just prior to the 37 °C incubation in step b, 1.50 ml of 0.01 M $MnCl_2$ should be added.

Deadenylylating activity of adenylyltransferase was measured as described by Mangum et al. (1973).

Protein determinations were by the biuret or Lowry methods (using bovine serum albumin as the reference protein) or from 280/260 absorbance ratios as outlined by Layne (1957), or from the 280 nm absorbance (see Results). A Cary Model 15 recording spectrophotometer was used for spectral measurements.

Growth of E. coli W. In small-scale growth studies, *E. coli* W cells were grown aerobically in a defined salts medium (Phares, 1971) containing glycerol as carbon source and either ammonia, glutamate, or proline as a nitrogen source.

[2] If glutamine synthetase is isolated by the method of Miller et al. (1974), which employs a zinc-induced paracrystalline aggregation, the enzyme subsequently must be freed of zinc in order to be adenylylated. The final acetone and acid ammonium sulfate steps (with Mn^{2+} present) of the purification procedure of Woolfolk et al. (1966) are partially effective in removing zinc (Hunt et al., 1975). Residual Zn^{2+} and inhibitory Mn^{2+} can be removed from glutamine synthetase by dialysis against 0.01 M imidazole–0.01 M K_2Mg-EDTA buffer (pH 7.1) and then against 0.01 M imidazole–0.01 M $MgCl_2$ buffer at pH 7.4.

[3] The radioactive assay under the conditions specified (Hennig and Ginsburg, 1971) gives a reliable measurement of adenylyltransferase activity in crude enzyme fractions since it is not influenced by the presence of either the P_{II} protein or glutamine synthetase in the ATase fraction. The assay conditions for measuring P_{IIA}-stimulated adenylylation are given in the legend to Figure 5.

[4] Since a subsaturating amount of glutamine synthetase is used in the colorimetric assay, the adenylyltransferase fraction should be free of GS_0 activity. Alternatively, GS_0 activity in the adenylyltransferase fraction can be assayed separately and the glutamine synthetase level in the assay then adjusted to give $A_{540nm} \simeq 1.0$.

Total growth was limited by the amount of either carbon or nitrogen added to the medium. Cells were harvested at different growth stages, suspended in 2 volumes/weight of cells in buffer (25 mM KPO_4, pH 7.8, 3 mM $MgCl_2$, and 1 mM β-mercaptoethanol), and ruptured by passage through a French pressure cell (Aminco) at ~12 000 psi. After centrifugation, the crude extract supernatant was assayed for adenylyltransferase activity using the radioactive incorporation assay.[3] The enzyme appeared to be constitutive as reported previously (Wohlhueter et al., 1973); optimal specific activities (~3.0–3.5 U/mg) were obtained during late log phase of growth on glycerol–glutamate medium.

Large-scale growth of *E. coli* W for purification of adenylyltransferase was in a 350-l. fermentor according to Phares (1971) with a growth medium (adjusted to pH 7) of 21.4 mM K_2HPO_4, 4.2 mM KH_2PO_4, 7 mM Na_2SO_4, 0.77 mM $MgSO_4$, trace metals (Phares, 1971), 308 mM glycerol, and 42 mM L-glutamate (glycerol levels should be at least fourfold those of glutamate). The pH during growth was maintained at pH 6.7 with 5% H_2SO_4; silicon antifoam (Dow Corning) was used (0.43 g/l.). Cells were harvested in late log phase, using a Sharples (Type A516P) continuous flow, water-cooled centrifuge at 15 000 rpm. Yields were ~5 kg cell paste for this level of glutamate (64% of theoretical) and were either used immediately or frozen in liquid nitrogen and stored at −20 °C in sealed containers until needed. For cell disruption, cells were suspended in ~2 volume/weight of buffer (25 mM KPO_4, pH 7.8, 3 mM $MgCl_2$, 1 mM β-mercaptoethanol, and 10 mM PMSF[5] with a Waring commercial blender (Model 99-215) run at low speed, and the suspension was filtered through cheese cloth and passed through a Mouton-Gaulin homogenizer (jacketed with ice–water) at ~12 000 psi.

Enzyme Purification Equipment. Centrifugations (unless otherwise indicated) utilized Sorvall RC-2B refrigerated centrifuges equipped with SZ-14 and standard rotors. Measurements of pH were with a Radiometer pH meter (Model 26). Chromatography columns (Pharmacia) were equipped with adjustable flow adaptors. Gradients were generated with an LKB Ultrograd attached to a peristaltic pump (Buchler). Column effluents were monitored with a conductivity meter (Radiometer Type CDM2c equipped with a flow-through 2.6 cm cell) and with an ultraviolet absorbance recorder at 280 nm (LKB Uvicord III attached to a Varian A-25 two-channel recorder). A LKB Model 7000 fraction collector was used. Gel columns were eluted in the upward direction; a 15% glycerol solution was applied immediately after the sample to retain a sharp protein boundary during application. Cellulose acetate electrophoresis (with a Shandon apparatus SAE-3225) was used to determine optimum conditions for purification steps.

Polyacrylamide gel electrophoresis was performed essentially by the method of Davis (1964), using a Büchler apparatus and a Shandon power supply. Ammonium persulfate and TEMED were used as cross-linking catalysts. Stacking gels were omitted; instead, lower sample ionic strength was used to sharpen protein bands (Hjertén et al., 1965). Acrylamide to bisacrylamide concentrations were used in ratios of 30:1 for electrophoresis of the native protein at 4 °C (pH 7.8) and of 37:1 for electrophoresis of the protein in sodium dodecyl sulfate (Shapiro et al., 1967; Weber and Osborn, 1969). Protein was stained by immersion overnight in a 0.1% Coomassie blue solution (acetic acid–ethanol–water, 10:45:45 by volume) followed by several changes of gel destaining solution (acetic acid–ethanol–water, 25:65:20 by volume).

Molecular weight determinations of native adenylyltransferase were by the method of Hedrick and Smith (1968) using the Tes-Bis-Tris buffer system at pH 7.8 (Rodbard and Chramback, 1971). Ovalbumin and bovine serum albumin (monomer and dimer) were used as reference proteins. Samples of adenylyltransferase and reference proteins (20–25 μg each) were applied in 15% glycerol, 1 mM thioglycolate, and 10 mM KPO_4 (pH 7.6) with 1 μl of 0.1% bromphenol blue added as a tracking dye. Gels of 6, 7, 8, and 10% acrylamide were run at 4 °C for 50–60 min at 3 mA/gel. Thioglycolate (10 mM) was incorporated into the upper reservoir buffer during the run. For enzyme activity determinations, the gel was sliced longitudinally (with a Canalco gel slicer) in the cold immediately after the electrophoresis of adenylyltransferase (~20 U/gel); one-half was stained for protein and the other half was sliced into 1.5-mm segments with a horizontal Canalco gel slicer. Each 1.5-mm slice was incubated at 4 °C for 2–4 h with 0.10 ml of 10 mM Tris buffer (pH 8), and then an aliquot was assayed for adenylyltransferase activity by the colorimetric assay (see above). The location of enzyme activity could be used to identify the adenylyltransferase protein band on the stained half of the gel for an estimate of purity.

Subunit molecular weight determinations in gels containing sodium dodecyl sulfate were performed after subjecting reference proteins and the adenylyltransferase to one of the following denaturation conditions: (1) incubation in 1% sodium dodecyl sulfate, 0.1 M β-mercaptoethanol, 0.025 M $NaPO_4$ (pH 7.1), and 15% glycerol for 3 h at 37 °C; (2) incubation with 6 M guanidine hydrochloride, 0.1 M β-mercaptoethanol, 1% sodium dodecyl sulfate, and 0.05 M $NaPO_4$ for 3 h at 50 °C; (3) reaction with threefold excess of iodoacetate in 6 M guanidine hydrochloride and 0.3 M triethanolamine (pH 8.5) at 50 °C for 30 min; (4) removal of phosphate and Mg^{2+} by dialysis against 2 mM EDTA and 10 mM Tris (pH 7.2) and then (a) incubation of an aliquot under the conditions of (1) above or (b) denaturation of an aliquot with 6 M urea. The denatured samples were dialyzed against 0.1% sodium dodecyl sulfate, 0.1 M β-mercaptoethanol, and 0.04 M $NaPO_4$ (pH 7.1) before electrophoresis at room temperature (~190 min at 5 mA/gel) in the same buffer (Weber and Osborn, 1969). The reference proteins used in these studies were: bovine serum albumin (containing some covalently linked dimers), phosphorylase *a*, *E. coli* glutamine synthetase, and muscle aldolase; see Klotz and Darnell (1969) for subunit molecular weights.

The cross-linking reagent, dimethyl suberimidate, was also used as described by Davies and Stark (1970) to determine the enzyme subunit structure. Adenylyltransferase and reference proteins (muscle aldolase, hemoglobin, muscle enolase, phosphorylase *a*, and myoglobin) were each incubated at a concentration of 2 mg/ml in 0.12 M triethanolamine (pH 8.5) to which dimethyl suberimidate (2.5 mg/ml) had been freshly added. After 3 h at room temperature, noncross-linked oligomeric proteins were dissociated by adding an equal volume of a mixture containing 2% sodium dodecyl sulfate, 0.2 M β-mercaptoethanol, and 0.05 M $NaPO_4$ (pH 7.1) and incubating for 16 h at 37 °C. Subse-

[5] Sometimes the cell suspension is acidic due to glycolysis occurring during cell harvesting. If so, extracts after cell rupture will also be acidic. To avoid this, the pH of the cell suspension should be checked before adding $MgCl_2$ and adjusted to pH 7 with 1 N KOH if necessary.

quent electrophoresis was for 4 h at 6 mA/gel with 5 or 7.5% gels and the pH 8.5 buffer of Davies and Stark (1970).

Isoelectric focusing of the purified adenylyltransferase was performed as described by Vesterberg (1971), using gradients of pH 3–10 and 0–50% glycerol.

Sample Preparation for Physical-Chemical Studies. The purified adenylyltransferase from this study (4600 U/mg; 4.7 mg/ml) was dialyzed successively at 4 °C for 6 and 19 h against 1000 × volumes of 0.1 M KPO_4–0.001 M $MgCl_2$ buffer at pH 7.83, which contained also 0.001 M β-mercaptoethanol in the initial dialysate. The final dialysate (ρ = 1.0113 and η/η_0 = 1.0432 at 25 °C) was used for subsequent dilutions of the enzyme. For optical work, solutions were clarified by a passage through a 0.45 μ Millipore filter. For studies of the denatured enzyme, the protein in 6.0 M guanidine hydrochloride was dialyzed against 6.0 M guanidine hydrochloride containing 0.05 M KPO_4 (pH ~5.2) for 66 h at room temperature. The density values of Kawahara and Tanford (1966) were used in the preparation of and in calculations involving 6.0 M guanidine hydrochloride solutions.

In addition, the lower molecular weight form of the enzyme reported by Hennig and Ginsburg (1971) was purified to electrophoretic homogeneity (2050 U/mg; see Results) and was dialyzed against 0.01 M $NaPO_4$, 0.1 M NaCl, 10^{-4} M EDTA, and 10^{-3} M β-mercaptoethanol buffer at pH 7.6 (ρ = 1.0025 at 25 °C). A sedimentation equilibrium run at 4 °C (using absorption optics at 280 nm) with this protein sample was performed as described below at speeds of 10 000 rpm (43 h) and 24 000 rpm (27 h).

Ultracentrifugation. A Beckman Model E ultracentrifuge was used, which was equipped with a rotor temperature and control unit, phase plate, schlieren, interference and absorption optics; the photoelectric scanner with multiplexer was connected to a Honeywell Electronik Model 194 recorder to expand the tracings. A two-place An-D rotor was used. Instrument calibrations, schlieren photographs, and viscosity and density measurements were as previously described (Shapiro and Ginsburg, 1968).

In sedimentation velocity experiments at 7–9 °C, protein concentrations of 4.7 to 0.7 mg/ml were run at 40 000 rpm for 1–2 h using a 12-mm cell with either a Kel F double sector or an aluminum-filled epoxy capillary synthetic boundary cell centerpiece. The observed sedimentation coefficients were corrected to values corresponding to a solvent with the viscosity and density of water at 20 °C ($s_{20,w}$).

For molecular weight determinations, low speed and meniscus depletion sedimentation equilibrium methods were used (Chervenka, 1969). Double sector Kel F cells (12 mm), equipped with sapphire windows, contained short liquid columns (130 μl) of sample and dialysate. For the native enzyme (at initial loading concentrations of 0.17–0.63 mg/ml), absorption or interference optics were used and the rotor was maintained at 4.2–5.9 °C and at speeds of 5200 (50 h), 16 000 (46 h), or 17 000 rpm (43 h). Only absorption optics were used for the protein in guanidine hydrochloride. Baseline corrections were made from scans at 340 nm (absorption runs) or from low speed interference fringe patterns obtained at the beginning of the run. Calculations of apparent weight average molecular weights were from slopes of linear plots of corrected ln A_{280nm} data (or ln A_{290nm} or fringe displacement data; Yphantis, 1964) vs. r^2 (the square of the radial distance from the center of rotation).

$$\sigma_w = \frac{\partial \ln A_{280}}{\partial r^2/2} = \frac{M_{w,app}(1 - \bar{V}\rho)\omega^2}{RT} \quad (3)$$

$M_{w,app}$ is the apparent weight average molecular weight of the macromolecular species, $\bar{V}$ is its partial specific volume, ρ is the solution density, ω is the angular rotor speed, R is the gas constant, and T is the absolute temperature.

High speed sedimentation equilibrium data were analyzed also by the computer program of Roark and Yphantis (1969), using an imposed error of 0.01 optical density units for absorption runs. The ideal moment M_{y1} is that derived by these workers (Roark and Yphantis, 1969; Yphantis and Roark, 1972) from the standard moments M_w and M_N (apparent weight and number average molecular weights, respectively), by which treatment the second virial coefficient is eliminated:

$$\left[\frac{1}{M_{y1}(c)} \equiv \frac{d(1/cM_N)}{d(1/c)} = \frac{2}{M_N(c)} - \frac{1}{M_W(c)}\right]$$

where c is the protein concentration.

Amino Acid Analyses. Protein samples (~0.1 mg each containing 0.025 μmol of norleucine as an internal standard) were hydrolyzed in 6 N HCl in evacuated tubes at 110 °C for 24, 48, or 72 h. Samples were analyzed on a Beckman Model 121 amino acid analyzer equipped with an Infotronics automatic digital integrator (Model CRS-210). Half-cystine content was determined by a modification of the method of Crestfield et al. (1963). Duplicate samples (each 0.11 mg of protein in 1.25 ml of 6 M guanidine hydrochloride, 0.11 M β-mercaptoethanol, 0.28 M Tris, pH 8.6) were incubated for 30 min at room temperature in the dark. Alkylation was initiated by adding 26.5 mg of iodoacetic acid in 0.1 ml of 1 M NaOH. After 30 min at room temperature, the alkylation reaction was stopped by adding 10 μl of 14.3 M β-mercaptoethanol, diluting fourfold with water, and placing the mixture in a boiling water bath for 5 min to precipitate the protein. After cooling, the samples were centrifuged for 10 min at 2600 rpm in a Sorvall GLC-1 centrifuge, the supernatants were decanted, and the precipitated protein was washed three times with 1 ml of water by centrifugation and decantation as before. The samples then were lyophilized and hydrolyzed in vacuo in 6 N HCl at 110 °C for 24 h. The amount of *S*-carboxymethylcysteine in each sample was determined on the amino acid analyzer.

Fluorescence emission spectra were measured with a Hitachi Perkin-Elmer MPF-2A spectrophotometer equipped with a voltage offset circuit and a Hewlett-Packard 700 AB X–Y recorder. For measurements of intrinsic typtophanyl fluorescence (Guilbault, 1973), excitation was at 300 nm (4.0 slit) and emission spectra were scanned from 310 to 460 nm with a 9.0 nm slit setting. Samples (1.5 ml) were thermostated at 20.0 °C by circulating refrigerated water from a P.M. Tamson Model PBC-4 bath (Neslab Instruments, Inc.) through the cell block of the fluorometer. A solution of L-tryptophan (3–6 μM), prepared in the same buffer and approximately matching the fluorescence of the protein solution, was used as an internal standard for each set of experiments. Fluorescence changes produced by glutamine (15 mM), ATP (1 mM), and α-ketoglutarate (9 mM) were measured separately with the protein and free tryptophan solutions. Results are expressed as a net change in fluorescence, which is equal to the measured fluorescence change in the protein solution *minus* the fluorescence change in the solution of free tryptophan.

Results

Purification of Adenylyltransferase. All steps were performed at 4 °C. For temporary enzyme storage between steps (after step 3 below), pooled column fractions were quickly frozen in liquid nitrogen and placed at −80 °C. Unless indicated otherwise, KPO_4 (25–400 mM, pH 7.6) buffers containing 1 mM $MgCl_2$ and 1 mM β-mercaptoethanol were used. The results are summarized in Table I.

Step 1: Preparation of Crude Extract. After cell rupture (see Methods),[5] the extract was centrifuged.

Step 2: Streptomycin Precipitation. Streptomycin sulfate (10% by volume of a freshly prepared 10% solution) was added in two equal stages to the supernatant from step 1. After making the extract 0.5% in streptomycin, the solution was centrifuged; the supernatant then was made 1% in streptomycin and centrifuged again.[6]

Step 3: First Chromatography on DEAE-Cellulose. The supernatant from step 2 was chromatographed in two stages: a batch procedure followed by a column step. For the batch procedure, ~2.5 l. of DEAE (equilibrated with 50 mM KPO_4 buffer at pH 7.6) was rapidly collected in a 3-l. scintered glass (coarse) filter funnel connected to a 4-l. filter flask; suction was applied and care was taken not to allow the DEAE to dry. (Settled bed volume ≃ 15 × 15 cm.) The DEAE then was stirred into the streptomycin sulfate supernatant, equilibrated for 15 min with stirring, and the DEAE collected in the funnel as before. Using suction and keeping a small liquid head, sequential washes with 25 mM KPO_4 (4 l.) and 10 l. of 0.10 M KPO_4 (without $MgCl_2$) were followed by an elution of the adenylyltransferase activity with 8 l. of 0.4 M KPO_4 (without $MgCl_2$). To the latter eluent was added a final wash with 3 l. of 25 mM KPO_4. For the column procedure, the effluent containing the adenylyltransferase activity was diluted with water to a conductivity equivalent to 0.1 M KPO_4 buffer (pH 7.6) and was made 1 mM in $MgCl_2$ concentration. To this diluted effluent, ~1.3 l. of fresh DEAE was added with stirring and then the DEAE was collected on the funnel as before. The DEAE (now green) was removed from the funnel, mixed with 50 mM KPO_4 buffer, and poured onto a 5-cm high bed of DEAE in a column (10 cm in diameter). The column was eluted at a flow rate of ~900 ml/h with a linear gradient from 100 to 400 mM KPO_4 buffer (8 l. total). A large protein peak was eluted as the gradient began, which was followed by the adenylyltransferase peak at 200–275 mM KPO_4 (yellow-green effluent); the latter fractions were pooled (Table I).

Step 4: Chromatography on Aminohexylagarose.[7] The pooled fraction from step 3 was diluted with 4 volumes of water to a conductivity equivalent to ~50 mM KPO_4 (pH 7.6), the $MgCl_2$ concentration was adjusted to 1 mM, and then the fraction was pumped onto an AH-Sepharose 4B column (10 cm diameter × 5 cm) which had been equilibrated with 50 mM KPO_4 buffer. After washing the column with 50 mM KPO_4 buffer, a linear KPO_4 gradient (4 l. of 50–250 mM KPO_4) was applied. A large protein peak (green) began to be eluted at 85 mM KPO_4; the adenylyltransferase activity was eluted on the descending portion of the green protein peak at 100–140 mM KPO_4. The enzyme peak fractions were pooled (Table I).

Table I: Purification of *E. coli* Adenylyltransferase.[a]

Step[b]	Volume (ml)	Enzyme Activity (Units × 10^{-3})[c]	Units/mg of Protein
(1) Crude extract	12 600	1 072	2.1
(2) Streptomycin sulfate supernatant	12 500	543[d]	2.3
(3) First DEAE-cellulose chromatography	2 175	407	81.2
(4) AH-Sepharose 4B chromatography	1 750	348	169
(5) Second DEAE-cellulose chromatography	206	145[e]	485
(6) Hydroxyapatite chromatography	87	78	1 074
(7) Agarose (1.5m), I-II	34	78	1 860
(8) Ammonium sulfate fractionation (0-42%)	6.5	72	2 557
(9) Agarose (1.5m), III-IV	2.4	29.4	4 600

[a] *E. coli* W grown to late log phase on glycerol–glutamate medium (see Methods) and freshly harvested (yield 5.15 kg). [b] See Results section. [c] One unit of enzyme catalyzes the incorporation of 1 nmol of [^{14}C]AMP per minute at 37 °C into unadenylylated glutamine synthetase. [d] The low pH of the crude extract in step 1 (pH 6) and the unadjusted pH of the streptomycin sulfate solution were responsible for the activity loss in this step (see text footnotes 6 and 7). [e] Most of this activity loss occurred because 1 mM $MgCl_2$ was not included in the dialysates of step 5.

Step 5: Second Chromatography on DEAE-Cellulose. The pooled fraction from step 4 was diluted with water to a conductivity equivalent to 100 mM KPO_4 buffer (pH 7.6), the $MgCl_2$ concentration was adjusted to 1 mM, and then the fraction was applied to a DEAE column (5 × 14 cm) at ~600 ml/h. The column was washed with 100 mM KPO_4 buffer and then eluted with a linear gradient (4 l. of 100–300 mM KPO_4). Again, the elution profile consisted of a large protein peak with the trailing fractions containing the adenylyltransferase activity. The pooled adenylyltransferase fraction was diluted with one-half volume of water and concentrated by a rapid passage over a DEAE column (5 cm diameter × 3 cm). The protein (including adenylyltransferase) was eluted sharply with 400 mM KPO_4 buffer and then was dialyzed against two changes (4 and 16 l.) of 5 mM KPO_4 buffer (see Table I) in preparation for step 6.

Step 6: Chromatography on Hydroxyapatite. The dialyzed fraction from step 5 was pumped onto a column of hydroxyapatite (2.5 × 25 cm) at a flow rate of 30 ml/h. The column was eluted stepwise with each phosphate concentration held constant for the number of column bed volumes indicated: a 5 mM KPO_4 (2 volumes) buffer wash was followed by 11 mM KPO_4 (3 volumes), 20 mM KPO_4 (2 volumes), 22 mM KPO_4 (1 volume), 25 mM KPO_4 (4 volumes), 33 mM KPO_4 (5 volumes), 37 mM KPO_4 (4 volumes), and 40 mM KPO_4 (2 volumes) buffers. A final wash with 100 mM KPO_4 regenerated the column. A protein

[6] In later purifications of the enzyme, it was found that the activity loss recorded in Table I for step 2 can be prevented completely by maintaining the pH at pH 7; i.e., the extract from step 1 and the 10% streptomycin solution should be adjusted to pH 7 before proceeding to step 2. A single addition of streptomycin sulfate (10% by volume) to the extract (pH 7) also results in a loss (≥30%) of adenylyltransferase activity (Ginsburg, 1970).

[7] Previous studies (Shaltiel, 1975; Shaltiel et al., 1975) showed that chromatography on ω-aminohexylagarose (AH-Sepharose) separated adenylyltransferase, glutamine synthetase, and the regulatory P_{II} protein.

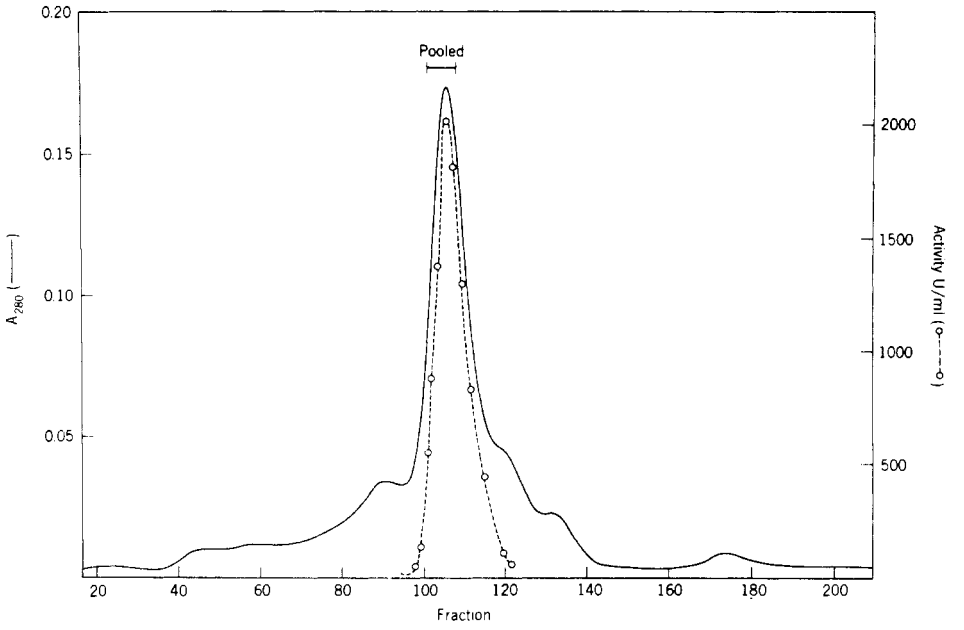

FIGURE 1: Elution profile for the Agarose III column (2.5 × 90 cm) step of the purification procedure for adenylyltransferase (see text). The column was equilibrated and eluted with 0.1 M KPO_4–0.001 M $MgCl_2$ buffer at pH 7.6 at 60 ml/h upward flow rate. The enzyme fraction from step 8 (28 mg in 6.5 ml) was applied; 2-ml fractions were collected. The adenylyltransferase activity (O - - - O) was pooled as indicated (fractions 100–106); fractions 107–115 were combined for the subsequent Agarose IV column run in step 9.

peak was eluted immediately after each increase in KPO_4 concentration. Almost equal adenylyltransferase activity appeared in both the 25 mM and 33 mM KPO_4 buffer elutions; at the 25 mM phosphate level, the enzyme eluted after about 1.5 bed volumes and tailed until the 33 mM KPO_4 buffer sharply eluted the remaining adenylyltransferase activity. After combining active fractions, the pooled effluent was concentrated on a small column of AH-Sepharose 4B (1.6 × 8 cm), using 200 mM KPO_4 buffer for protein elution (Table I).

Step 7: Agarose I–II. The sample from step 6 was concentrated further by adding 2 volumes of saturated ammonium sulfate, centrifuging, and dissolving the precipitate (green) with 20 mM KPO_4 buffer. The dissolved precipitate was applied to an Agarose (1.5m) column (2.5 × 90 cm) which had been equilibrated with the same buffer (upward flow rate of 60 ml/h; 2-ml fractions collected). Two main protein peaks were observed: a small peak eluted first (containing the green protein) and was partially overlapped by a second larger peak (containing the adenylyltransferase). The side and central portions of the main enzyme peak were pooled separately. The side fractions were concentrated by an ammonium sulfate precipitation and rechromatographed in the same Agarose 1.5m column as above. The majority of adenylyltransferase activity from the two Agarose column runs was combined and concentrated by a passage through a small AH-Sepharose 4B column (1.6 × 2 cm) as in step 6.

Step 8: Ammonium Sulfate Fractionation.[8] To the fraction from step 7, 42% by volume of saturated ammonium sulfate was added slowly with stirring. After 30 min, the solution was centrifuged and the precipitate saved. To the supernatant, ammonium sulfate was added to a final 62% saturation; after 30 min the precipitate (yellow green) was collected by centrifugation and dissolved in 20 mM KPO_4 buffer. Ammonium sulfate (42% by volume) then was added. Again, the precipitate was collected by centrifugation. The two 42% ammonium sulfate precipitates were dissolved in 20 mM KPO_4 buffer and combined (Table I). This fraction had a pale green color (409 nm absorbance) due to an impurity present.

Step 9: Agarose III–IV. Step 7 was repeated; the elution profile of the Agarose III column run is shown in Figure 1. The main peak fractions had a constant specific activity and were electrophoretically pure. These fractions were pooled (as shown) and saved, while the other fractions containing significant amounts of enzyme were combined, precipitated with 2 volumes of ammonium sulfate, and rechromatographed on Agarose (Agarose IV). This time, a single protein peak with a small tailing shoulder was observed. Purity was checked as above and only electrophoretically homogeneous fractions were pooled. The homogeneous protein pools from Agarose III and IV column runs were precipitated with 2 volumes of ammonium sulfate, dissolved in 20 mM KPO_4 buffer (final protein concentration $\geq$ 1 mg/ml), and dialyzed (see Methods). The enzyme was stored at −80 °C after quick freezing with liquid nitrogen.

Enzyme Stability. The stability of the purified adenylyltransferase was tested under various conditions. In KPO_4 buffers (10–100 mM, pH 7.6) containing 1 mM $MgCl_2$, the enzyme was stable for months when stored at −80 °C at concentrations above 0.1 mg/ml. (At 8 μg/ml, the activity was stable overnight at −80 °C but decreased 60–70% in 14 days at −80 °C.) The enzyme also was stable for 12 days at 0 to 4 °C at concentrations above 1 mg/ml. Without $MgCl_2$ present in phosphate buffers, the enzyme was much less stable. Half-life values for the enzyme at low concentration (8 μg/ml) in different buffers were obtained from first-order plots of the decay of enzyme activity. In 10 mM KPO_4–1

[8] With dilute protein solutions, a more efficient extraction of the green protein fraction with ammonium sulfate is as follows (with all solutions containing 50 mM KPO_4, 1 mM $MgCl_2$, and 1 mM β-mercaptoethanol, pH 7.6). The adenylyltransferase fraction initially is concentrated by adding 70% by volume of a saturated ammonium sulfate solution (4 °C) and collecting the precipitate by centrifugation. The precipitate then is extracted twice with 40% ammonium sulfate to solubilize the green protein fraction and twice with 30% ammonium sulfate to solubilize the adenylyltransferase. The enzyme then is precipitated by adding an equal volume of saturated ammonium sulfate.

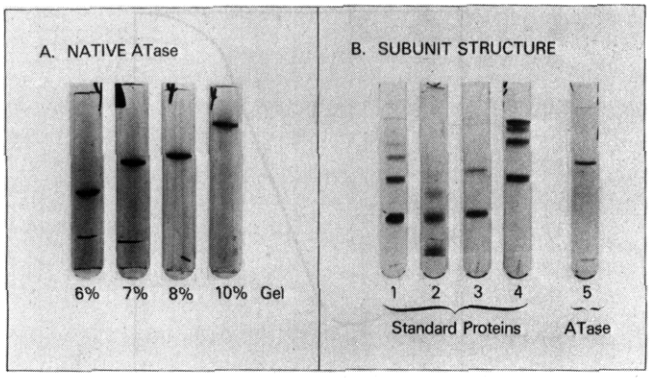

FIGURE 2: Polyacrylamide gel electrophoresis studies. (A) Stained gels (6, 7, 8, and 10%) of the native enzyme (20 μg/gel) are shown. (The dye marker is visible in most of the photographs.) (B) Various oligomeric proteins (1–4) and adenylyltransferase (5) were amidinated with a bifunctional, cross-linking reagent (dimethyl suberimidate) before electrophoresis on polyacrylamide gels (5%) in the presence of sodium dodecyl sulfate (see Methods). The reference proteins (with each molecular weight and number of subunits given in parentheses; Klotz and Darnell, 1969) were: (1) muscle aldolase (160 000; 4); (2) hemoglobin (64 500; 4); (3) muscle enolase (82 000; 2); and (4) phosphorylase a (370 000; 4).

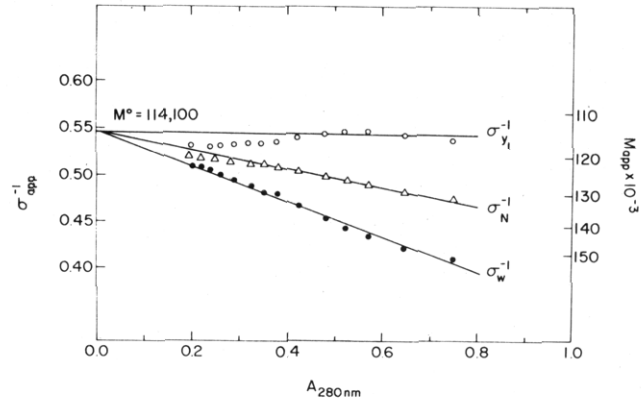

FIGURE 3: Sedimentation equilibrium of adenylyltransferase in 6 M guanidine hydrochloride. The protein (0.20 mg per ml of 6 M guanidine hydrochloride–0.05 M KPO_4 at pH ~5.2) was centrifuged at 15 000 rpm (20.9 °C) for 25 h after 47 at 17 000 rpm. The data were analyzed by the computer program of Roark and Yphantis (1969), with an imposed error of ±0.01 optical density unit. Point average reciprocal σ_W, σ_N, and the σ_{y_1} ideal moment of Yphantis and Roark (1972) are plotted vs. the protein absorption at 280 nm in the centrifuge cell (see Methods). The right ordinate shows corresponding apparent molecular weight values.

mM $MgCl_2$ buffer at pH 7.6, the half-life of enzyme activity at 0 or 25 °C was 192 or 46 h, respectively. In Tris-HCl (pH 8.0) or in imidazole-HCl (pH 7.6) buffers (with or without $MgCl_2$ present), the half-life of the enzyme activity was only about 4 h at 0 °C. Adenylyltransferase is therefore considerably less stable in Tris or imidazole buffer than in a magnesium phosphate buffer.

Physical and Chemical Properties. Polyacrylamide gel electrophoresis studies with the native enzyme are shown in the photographs of Figure 2A. The location of adenylyltransferase activity on each gel corresponded exactly to the band of stained protein. These studies indicate that the purified adenylyltransferase was homogeneous with respect to electrophoretic mobility. The molecular weight of adenylyltransferase was determined to be 112 000 from the relative mobility of the enzyme and reference proteins on polyacrylamide gels (Hedrick and Smith, 1968). In similar experiments with much less pure adenylyltransferase fractions, the relative mobility of enzyme activity on gels gave identical results. A larger molecular weight complex with associated adenylyltransferase activity in crude enzyme fractions, therefore, is not apparent on polyacrylamide gels.

Despite the use of harsh denaturing and dissociating conditions (see Methods), the purified protein stained as a single band after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The mobility of the denatured enzyme when compared with electrophoretic mobilities of reference proteins (see Methods) corresponded to that of a protein of molecular weight 105 000 in 5% gels, 119 000 in 7.5% gels, and 100 000 in 10% gels. These data indicate that adenylyltransferase is composed of a single polypeptide chain. This conclusion is corroborated by the results obtained after treating the enzyme with the cross-linking reagent, dimethyl suberimidate (Figure 2B).

Davies and Stark (1970) showed that amidination of various oligomeric proteins with dimethyl suberimidate yields species which can be resolved by electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate. Each set of resolved species had molecular weights equal to integral multiples of the protomer molecular weight. For oligomers composed of identical subunits, the number of principal species was observed to equal the number of protomers in the oligomer. In the present studies, amidination of myoglobin (results not shown), muscle aldolase, muscle enolase, or phosphorylase *a* with dimethyl suberimidate (see gels 1, 3, and 4 of Figure 2B) gave the expected one, four, two, or four protein bands, respectively. Bovine hemoglobin (gel 2 of Figure 2B), which has a subunit structure of $\alpha_2\beta_2$ (Dayhoff, 1972), shows one faint and three distinct protein bands. Under the same conditions, amidination of adenylyltransferase (gel 5 in Figure 2B) yields a single protein band (with the mobility unchanged by amidination).

In an isoelectric focusing experiment, the adenylyltransferase activity and 280 nm absorbance peaks appeared sharply at pH 4.98.

Sedimentation equilibrium studies indicated that the purified adenylyltransferase was homogeneous with respect to size. From four separate sedimentation equilibrium experiments at different initial protein concentrations (0.17–0.62 mg/ml), the weight average molecular weight (M_w^0) was 115 000 ± 5000 at infinite dilution (assuming $\bar{V}$ = 0.73 ml/mg; see Table II below) and

$$\frac{1}{M_w(\text{app})} = 0.87\ (\pm 0.04) \times 10^{-5} + 0.4 \times 10^{-5}c \qquad (4)$$

where c is the initial concentration in mg/ml.

In sedimentation velocity studies of the native enzyme at different protein concentrations, a single, symmetrical boundary was observed with

$$s_{20,w} = 5.6 \pm 0.1S(1 - 0.044c) \qquad (5)$$

where c is the initial concentration in mg/ml. From the values of $s_{20,w}^0$ and M_w^0, the calculated diffusion coefficient ($D_{20,w}^0$) is 4.4 × 10^{-7} cm^2/s with a molar frictional ratio (f/f_0) of 1.52. These hydrodynamic values indicate that adenylyltransferase behaves as a somewhat asymmetric particle in solution.

A high speed sedimentation equilibrium study of the enzyme in 6 M guanidine hydrochloride is illustrated in Figure 3. Point average reciprocal apparent σ values (weight average, number average, and y_1 ideal moments of Yphantis and Roark, 1972; see Methods) from the concentration

Table II: The Amino Acid Composition of Adenylyltransferase.[a]

Amino Acid Residue	Residues per 115 000 Mol Wt	Integer Values
Lysine	37.5 ± 0.6	38
Histidine	20.9 ± 1.4	21
Arginine	78.3 ± 1.1	78
Aspartic acid	99.4 ± 1.6	99
Threonine	45.1 ± 0.8	45
Serine	60.6 ± 2.7	61
Glutamic acid	148.8 ± 2.4	149
Proline	41.4 ± 1.6	41
Glycine	77.8 ± 3.0	78
Alanine	101.7 ± 2.9	102
Half-cystine	8	8
Valine	69.0 ± 0.2	69
Methionine	7.9 ± 2.6	8
Isoleucine	47.6 ± 0.5	48
Leucine	117.1 ± 3.2	117
Tyrosine	21.5 ± 0.9	22
Phenylalanine	36.5 ± 1.9	37
Tryptophan	15[b]	15
Total:		1036

[a] Each value is the mean of six individually hydrolyzed protein samples (duplicates at 24, 48, and 72 h hydrolysis). The standard deviation from the mean for each amino acid residue is indicated; the overall precision was equal to ±3%. Serine and threonine values were obtained by least-squares extrapolations to zero time of hydrolysis. The 72-h value was used for isoleucine. Half-cystine was determined as *S*-carboxymethylcysteine (see Methods). [b] Tryptophan was determined from the ultraviolet absorption spectrum of the enzyme in 6 M guanidine hydrochloride by the method of Edelhoch (1967).

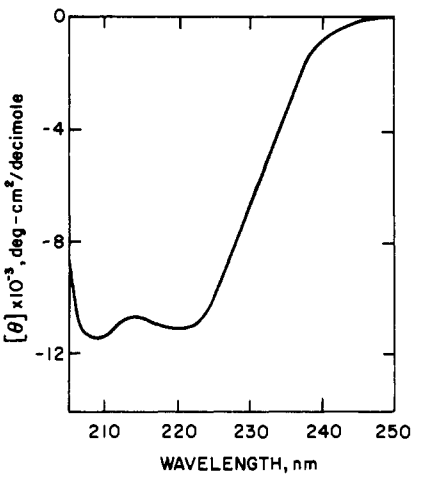

FIGURE 4: Circular dichroism spectrum of adenylyltransferase at 28 °C. The enzyme concentration was 0.30 mg per ml of 0.1 M KPO_4–0.001 M $MgCl_2$ buffer at pH 7.83, and a fused silica gel of 0.1-cm path length was used. Circular dichroism (CD) measurements from 300 to 200 nm were made with a Cary Model 60 recording spectropolarimeter, equipped with a Model 6001 CD attachment. The protein spectrum was corrected by an average of baseline spectra recorded with the cell filled with buffer alone just prior to and after measurements on the protein solution. The mean residue ellipticity is plotted as a function of wavelength.

gradient are plotted as a function of the protein concentration in the cell (A_{280nm}). The negative slopes of the σ_W^{-1} and σ_n^{-1} data in Figure 3B suggest that there was some self-association under these conditions that was corrected for by elimination of the second virial coefficient (zero slope of the $\sigma_{y_1}^{-1}$ data in Figure 3B; Roark and Yphantis, 1969). The concentration dependence of the apparent weight average or number average molecular weights is shown more clearly by the M_{app} values in Figure 3. The concentration independence of the ideal moment (y_1) of Yphantis and Roark (1972) within experimental error shows that the denatured enzyme is homogeneous (Roark and Yphantis, 1969). Most significantly, inverse sigma data for the enzyme in 6 M guanidine hydrochloride extrapolate to a molecular weight of 114 000 ± 2000 at infinite dilution (assuming $\bar{V}$ = 0.73 ml/g). These results, which confirm those already presented (see above), indicate that adenylyltransferase is a single polypeptide chain.

The amino acid composition of adenylyltransferase is given in Table II. The basic amino acid residues are ~55% of the acidic residues (glutamic plus aspartic acids). Although the number of amide groups was not determined, the acidic nature of the protein (pI = 5; see above) suggests that >50% of the carboxyl groups are free. The high arginine content precluded the use of tryptic digestion (even with lysyl ϵ-amino groups blocked) for peptide mapping. The value of eight half-cystine residues per enzyme molecule agrees with the number of titratable sulfhydryl groups reported by Wolf and Ebner (1972). Adenylyltransferase, therefore, contains no disulfide bridges. The partial specific volume ($\bar{V}$) from the amino acid composition (Table II) was calculated (Cohn and Edsall, 1943) to be 0.733 ml/g, assuming 55 residues each of asparagine and glutamine per enzyme molecule. Molecular weight calculations in eq 4 and Figure 3 (above) were based on a value of $\bar{V}$ = 0.73 ml/g.

The ultraviolet spectral and fluorescence (see below) properties of the enzyme relate to the tryptophanyl and tyrosyl residue content (Table II). Adenylyltransferase has a specific absorbance at 280 nm of $A_{1cm}^{1\%}$ = 10.5, using the biuret method for determining protein concentration (see Methods). The ultraviolet spectrum of the native enzyme has an absorbance maximum at 280 nm (A_{280nm}/A_{260nm} = 1.7) with a shoulder at 290 nm (A_{290nm}/A_{280nm} = 0.72) and an absorbance minimum at 251 nm.

The circular dichroism spectrum for the adenylyltransferase is illustrated in Figure 4. With a mean amino acid residue weight of 111 calculated from the data in Table II, the mean residue ellipticity [θ] at 208 nm under the conditions of Figure 4 is −11 200 (deg cm^2)/dmol. From this value, an α-helical content of 25% is estimated for adenylyltransferase by the method of Greenfield and Fasman (1969) which is based on an isosbestic point at 208 nm for the circular dichroism spectra of random coil and β-structures. With the calculated α-helical content and the circular dichroism spectrum of the enzyme shown in Figure 4, the standard curves of Greenfield and Fasman (1969) could be fit approximately with structural contents of ~28% β-pleated sheet and ~47% random-coil segments in the adenylyltransferase molecule. The rather large polypeptide chain of the enzyme molecule thus appears to have only ~53% structured segments.

Interactions of Adenylyltransferase with Effectors. Both adenylylation (eq 1) and deadenylylation (eq 2) reactions are catalyzed by adenylyltransferase in the presence of Mg^{2+} or Mn^{2+} (Anderson et al., 1970; Anderson and Stadtman, 1971; Brown et al., 1971). This was confirmed in the present studies with purified adenylyltransferase. A coupling between reactions 1 and 2, which would generate a futile cycle (Ginsburg and Stadtman, 1973), is prevented by an elaborate regulatory system in *E. coli*. This involves metabolite control of adenylyltransferase and an interaction of adenylyltransferase with the small regulatory P_{II} protein,

which exists in two interconvertible forms. Divalent cations also have specific roles in the regulatory mechanisms.

The data of Figure 5 illustrate that in the presence of a saturating amount of the P_{IIA} protein the Mg^{2+}-supported adenylyltransferase activity is extremely responsive toward activation by glutamine, whereas the Mn^{2+}-supported activity is almost unchanged by varying glutamine concentrations. With Mn^{2+} present, glutamine appears to have both activating and inhibitory effects; the small increase in activity at low glutamine concentrations was reproducible. In the absence of glutamine, the adenylyltransferase activity in the presence of a saturating amount of P_{IIA} protein is about eightfold greater in the Mn^{2+}- than in the Mg^{2+}-supported adenylylation reaction.

In experiments not illustrated, the P_{IIA} stimulation of adenylylation (i.e., the ratio of ATase activities: (ATase + P_{IIA})/ATase) was measured under the assay conditions of Figure 5. In a Mg^{2+}- or Mn^{2+}-supported assay without glutamine present, the stimulation by the P_{IIA} protein was ~3 or ~5, respectively. In a Mg^{2+}-supported assay, the P_{IIA} stimulation is the same with or without glutamine present. The P_{IIA}-stimulation of adenylylation was found to be pH independent in the range of pH 7.2–8.5. With or without the regulatory P_{IIA} protein present, the pH optimum of adenylylation is pH 8.0–8.2 in either a Mg^{2+}- or Mn^{2+}-supported reaction.

Adenylyltransferase was treated with increasing concentrations of urea or of a mercurial reagent (PMPS). Both adenylylating and deadenylylating activities of the enzyme decreased similarly during treatments with either denaturant. Exposure of the enzyme to 4 M urea or to 10^{-3} M PMPS for 15 min at 37 °C (with or without P_{II} protein present during this incubation) completely inactivated adenylyltransferase in catalyzing both adenylylating and deadenylylating reactions. (It should be noted that the P_{II} protein contains no sulfhydryl groups (Adler et al., 1975).) Dithiothreitol was effective in reversing the inactivation of adenylyltransferase caused by PMPS. Earlier studies of Hennig et al. (1970) suggested that the adenylylating and deadenylylating activities were affected differently by a mercurial modification of the enzyme. However, the deadenylylating assays of Hennig et al. (1970) contained dithiothreitol so that mercurial-induced inactivation of adenylyltransferase was reversed during the measurement of deadenylylation.

Wolf and Ebner (1972) showed that two to four of the eight sulfhydryl groups of adenylyltransferase could be titrated with DTNB without loss of adenylylating activity. A partial reaction of the enzyme with PMPS, however, causes some desensitization toward glutamine activation in a Mg^{2+}-supported adenylylation reaction. (Figure 5 only illustrates results for the P_{IIA}-stimulated adenylylation reaction; in the absence of the P_{II} protein, glutamine activation curves were threefold lower in amplitude but similar in shape for the Mg^{2+}-supported adenylyltransferase activity.) High concentrations of glutamine appear to reverse structural alterations in adenylyltransferase produced by sulfhydryl modification. In contrast, the Mn^{2+}-supported (P_{IIA}-stimulated) activity of the mercurial derivative is not restored by high concentrations of glutamine.

Since protein fluorescence is often a sensitive measure of the stabilization of different conformational states by ligands, various effectors of reaction 1 were tested to see whether or not these influenced the fluorescence of adenylyltransferase. Effectors of adenylylation that were tested

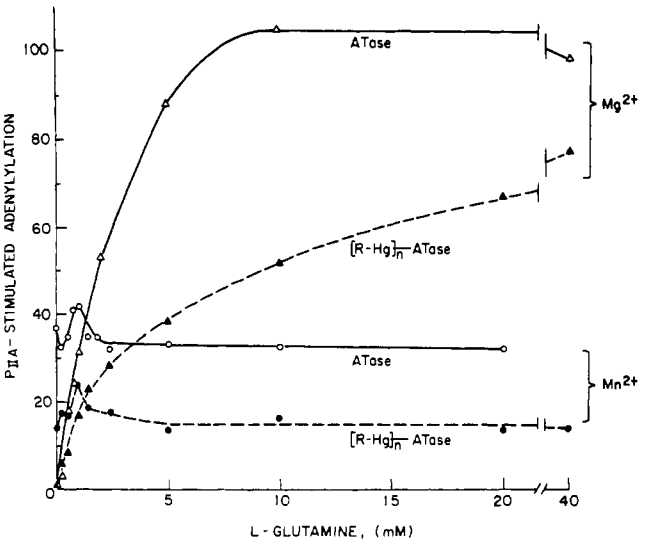

FIGURE 5: The effects of glutamine on adenylyltransferase-catalyzed (P_{IIA}-stimulated) adenylylation in the presence of Mg^{2+} or Mn^{2+} and a saturating amount of P_{IIA} protein. An adenylyltransferase fraction which had been pretreated with PMPS (10^{-4} M PMPS at pH 8 for 2 h at 0 °C) is indicated by $[R-Hg]_n$—ATase. Optimal assay conditions for [^{14}C]AMP incorporation into GS in a 50 μl reaction volume were: a buffer (pH 8.0) containing 50 mM imidazole, 50 mM 2-methylimidazole, and 50 mM 2,4-dimethylimidazole, 1 mM [^{14}C]ATP (10^6 cpm/μmol), 300 μg of GS_1, either 20 mM $MgCl_2$ with 0.3 μg of ATase or 5.4 mM $MnCl_2$ with 0.6 μg of ATase, a saturating amount of the regulatory P_{IIA} protein, and the indicated concentrations of L-glutamine. The ordinate values are expressed in units of adenylyltransferase activity (see Methods). The data of this figure were obtained in collaboration with Dr. E. J. Oliver.

included the activators, Mg^{2+}, ATP, glutamine, and the unmodified form of the regulatory P_{II} protein (Brown et al., 1971; Mangum et al., 1973), and the inhibitors, α-ketoglutarate (Ebner et al., 1970) and phosphate (Hennig and Ginsburg, 1971).

Figure 6 (curve 2) shows the intrinsic tryptophanyl residue fluorescence of the adenylyltransferase in phosphate–$MgCl_2$ buffer. When the enzyme was added either to the regulatory P_{II} protein, which by itself has a very low fluorescence yield (dashed curve 1 of Figure 6), or to 15 mM glutamine, an enhancement of fluorescence was observed. The net enhancement produced by a twofold molar excess of P_{II} protein (curve 3) or by a saturating level of glutamine (curve 4) was 8 or 20%, respectively, under these conditions. Glutamine also produced a slight red shift from 343 to 345 nm in the maximum of the emission spectrum of the adenylyltransferase. In other studies at the same pH and temperature (not illustrated), a buffer of 40 mM imidazole–1 mM $MgCl_2$ was used in order to approximate assay conditions (Brown et al., 1971). In these experiments with the adenylyltransferase, enhancements in the fluorescence at 345 nm were 10% by a twofold molar excess of P_{II} protein, 11% by 10 mM glutamine alone, and 22% by a mixture of both these effectors of the adenylylation reaction 1. Using an internal reference solution of L-tryptophan (see Methods), the addition of 1 mM ATP or of 9 mM α-ketoglutarate to the enzyme produced a net fluorescence change at 345 nm of +7 or −7%, respectively. In the presence of the regulatory P_{II} protein, however, 1 mM ATP had no apparent effect on the fluorescence of the adenylyltransferase. Orthophosphate (10 mM) also had no effect.

The intrinsic fluorescence of tryptophanyl residues of the adenylyltransferase is 2.0-fold higher than that of free tryp-

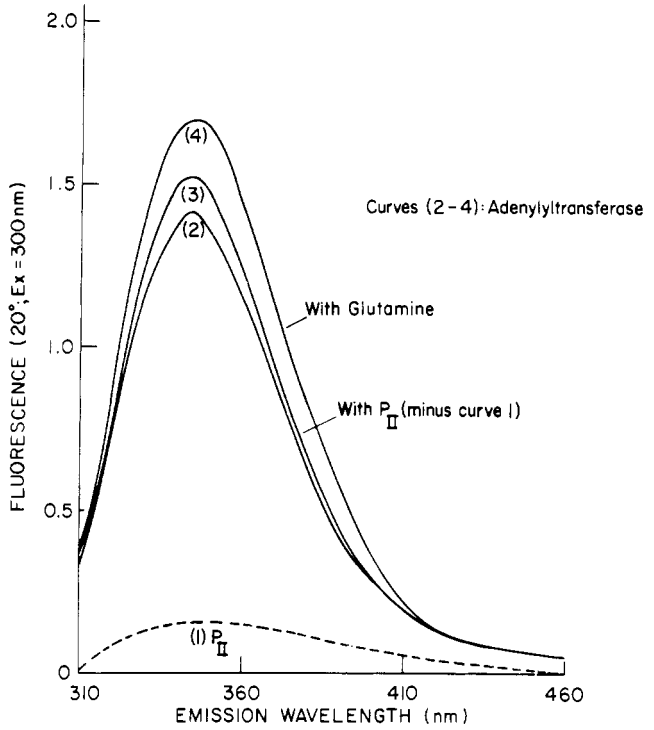

FIGURE 6: Fluorescence emission spectra at 20 °C with excitation at 300 nm. The solid curves (2–4) are spectra of 0.077 μM adenylyltransferase (8.9 μg/ml) and the dashed curve (1) is the spectrum of 0.15 μM regulatory P_{II} protein (5.7 μg/ml) in a buffer of 0.10 M KPO_4–0.001 M $MgCl_2$ at pH 7.74. Fluorescence emission spectra (curves 2–4) were obtained by adding adenylyltransferase (4600 U/mg) to buffer alone (curve 2), to buffer with a 1.9-fold molar excess of the regulatory P_{II} protein (resultant curve 3, with curve 1 for P_{II}-protein fluorescence already subtracted from the observed curve), and to buffer containing 15 mM L-glutamine (curve 4).

tophan. The low fluorescence yield of the regulatory P_{II} protein reflects the low tryptophanyl residue content of this protein which is approximately equal to 4 tryptophans per tetramer of molecular weight 45 600 (Adler et al., 1974, 1975). The fluorescence enhancement produced by the P_{II} protein (Figure 6), however, could involve changes in the microenvironment of tryptophanyl residues of the regulatory protein, of the adenylyltransferase, or of both. Glutamine synthetase, the substrate of the adenylyltransferase, has 36 equiv of tryptophanyl residues per mole (Hunt and Ginsburg, 1972) and, therefore, fluorescence studies with mixtures of these two proteins were not attempted. The fluorescence changes caused by the small ligands (glutamine, ATP, and α-ketoglutarate) are more easily interpreted as direct effects on a stabilization or destabilization of the conformation of the adenylyltransferase.

A Low-Molecular-Weight Adenylyltransferase. In previous studies in this laboratory (Hennig and Ginsburg, 1971), adenylylation activity was associated with a protein of about 70 000 molecular weight. This protein had no activity in P_{IID}-stimulated deadenylylation (reaction 2), but it had almost the same kinetic properties in the adenylylation reaction as did the higher molecular weight form of the adenylyltransferase isolated by Ebner et al. (1970). In other studies, it was shown that the larger adenylyltransferase was slowly converted during storage at 4 °C to a smaller protein that was active only in adenylylating glutamine synthetase (Hennig et al., 1970). Partially purified enzyme fractions which had been stored at 4 °C from these earlier studies were combined and purified to electrophoretic homogeneity in steps similar to those described above except that Sephadex G-100 was substituted for Agarose filtration. A molecular weight of 64 000 ± 4000 was determined in low and high speed sedimentation equilibrium studies (see Methods). This small adenylyltransferase had an activity of 2050 U/mg in a glutamine-activated, Mg^{2+}-supported, adenylylation reaction (see Methods under Radioactive Assay).

Discussion

Adenylyltransferase from *E. coli* W was shown here to be an asymmetric particle with a frictional ratio of 1.5 and a molecular weight of 115 000 (Figures 2 and 3). This molecular weight value is similar to that reported by Wolf et al. (1972) for the enzyme from *E. coli* B. In the present isoelectric focusing experiment, however, a single active protein band appeared at pH 5.0, whereas Wolf et al. (1972) found a predominant activity band at pH 4.2–4.3.

The adenylyltransferase isolated by Ebner et al. (1970) was unstable in imidazole buffer at pH 7.5; see above. Inactivation, which was accompanied by protein aggregation and a decrease in titratable sulfhydryl groups (Wolf et al., 1972), was prevented by the addition of bovine serum albumin to the enzyme. Wolf and co-workers (1972) proposed that the enzyme was stabilized by bovine serum albumin through a protein–protein interaction similar to that between adenylyltransferase and the regulatory P_{II} protein. It is known now, however, that adenylyltransferase, bovine serum albumin, and the P_{II} protein have about the same net charge, but that the P_{II} protein differs chemically and physically from bovine serum albumin (Adler et al., 1975). For example, the P_{II} regulatory protein from *E. coli* is a tetrameric protein of 46 000 molecular weight, contains no cysteine or cystine residues and can undergo uridylylation (Adler et al., 1975; Brown et al., 1971).

In the present study, the isolated adenylyltransferase was stabilized simply by buffers containing both magnesium and phosphate. This could be related to an earlier observation that adsorption of adenylyltransferase onto a manganese phosphate precipitate induced a conformation active in deadenylylation (Anderson and Stadtman, 1971). At higher concentrations of adenylylated glutamine synthetase, magnesium phosphate also is effective (Brown et al., 1971).

The discovery that adenylyltransferase is a single polypeptide chain means that the previously reported inactivation of the enzyme was not due to a mechanism involving subunit dissociation. Moreover, the previously isolated adenylyltransferase of about 64 000 molecular weight must have resulted from a proteolytic cleavage of the enzyme. The slow conversion of the enzyme to a smaller molecular weight species observed in the studies of Hennig et al. (1970) supports this conclusion. The low-molecular-weight adenylyltransferase, once formed, nevertheless was stable for months at 4 °C (Hennig and Ginsburg, 1971). A nuclease digestion step (at 37 °C) in the earlier purification procedure of Hennig and Ginsburg (1971) could have been responsible for such a proteolytic cleavage, with the protease either present in the cell extract or introduced inadvertently. In the present purification procedure, a protease inhibitor (PMSF) was included in the cell breakage buffer as a precaution. Preliminary attempts to produce a smaller active enzyme species by a limited digestion of adenylyltransferase with trypsin were unsuccessful.

The adenylyltransferase of 115 000 molecular weight catalyzes both adenylylation and deadenylylation of gluta-

mine synthetase (Anderson et al., 1970; Anderson and Stadtman, 1971; Brown et al., 1971). A simultaneous loss of both activities occurs upon treatment of the enzyme with a mercurial reagent (PMPS), with heat (Anderson et al., 1970), or with urea. The P_{II} protein has a regulatory role in both reactions; in its unmodified or uridylylated form, the P_{II} protein stimulates adenylylation (eq 1) or deadenylylation (eq 2), respectively. The stimulation of deadenylylation by $P_{IID(UMP)}$ is much greater than that of adenylylation by P_{IIA} (Brown et al., 1971). In fact, it was thought originally that the deadenylylation reaction (eq 2) absolutely required the regulatory P_{II} protein component (Shapiro, 1969). The stimulation of deadenylylation by $P_{IID(UMP)}$, therefore, is the most sensitive measure of the interaction between adenylyltransferase and the P_{II} protein. The inability of the previously isolated, low-molecular-weight adenylyltransferase to catalyze $P_{IID(UMP)}$-stimulated deadenylylation (Hennig et al., 1970) probably reflects a loss in the combining site of adenylyltransferase for the P_{II} protein. The cleavage of the adenylyltransferase, however, did not influence significantly the kinetic response of the small enzyme species in the adenylylation reaction to the positive effectors, Mg^{2+} (or Mn^{2+}), L-glutamine, ATP, and unadenylylated glutamine synthetase, or to the negative effector, α-ketoglutarate (Hennig and Ginsburg, 1971).

Fluorescence measurements with a hydrophobic probe (Holzer, 1969), as well as previous kinetic studies (Hennig and Ginsburg, 1971), indicated that adenylyltransferase has one or more allosteric sites for L-glutamine. Wolf and Ebner (1972) also found that the number of exposed sulfhydryl groups in adenylyltransferase titratable with DTNB was increased by positive effectors of adenylylation (Mg^{2+}, glutamine, glutamine synthetase, and ATP) and was decreased by inhibitors of adenylylation (α-ketoglutarate and 3-phosphoglycerate). In the present study, the tryptophanyl residue fluorescence of adenylyltransferase was enhanced by glutamine and ATP and was decreased by α-ketoglutarate. Thus, positive and negative effectors of adenylylation stabilize different conformations of adenylyltransferase. The fluorescent enhancements produced by glutamine and the P_{II} protein were additive. The fluorescence enhancement produced by the P_{II} protein, however, could involve tryptophanyl residue perturbations in both adenylyltransferase and the P_{II} protein. Glutamine, but not the regulatory P_{II} protein, caused a slight red shift in the fluorescence emission spectrum of adenylyltransferase. The fluorescent and kinetic results with the intact enzyme, together with the established catalytic properties of a smaller enzyme species, indicate that the activating sites for glutamine and for the P_{II} protein are separate. Nevertheless, the presence of the P_{IIA} protein in a Mg^{2+}-supported adenylylation reaction increases the glutamine stimulation about fourfold.

The large size of the polypeptide chain of adenylyltransferase is unusual. It was shown by Waterson and Konigsberg (1974) that the monomeric leucyl-tRNA synthetase from *E. coli* of 100 000 molecular weight contains extensive internal amino acid sequence homology. These authors suggested that this polypeptide chain had evolved through a process of gene duplication and fusion. Peptide mapping studies are necessary to establish whether or not the polypeptide chain of adenylyltransferase had a similar origin. Proteolytic digestions of adenylyltransferase with trypsin or chymotrypsin would yield too many peptides (Table II) to be resolved by peptide mapping techniques. Autoradiograph peptide mapping after radioactive labeling of lysyl, methionyl, or cysteinyl residues (as performed by Waterson and Konigsberg (1974) with tRNA synthetases) is possible and will be attempted.

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Cryoenzymology of Chymotrypsin: The Detection of Intermediates in the Catalysis of a Specific Anilide Substrate†

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ABSTRACT: The reaction between chymotrypsin and *N*-acetyl-L-phenylalanine *p*-nitroanilide has been studied at subzero temperatures in fluid aqueous dimethyl sulfoxide solvent. Following initiation of the reaction at temperatures as low as −90 °C, a series of four reactions prior to the normal rate-limiting step (acylation) was detected spectrophotometrically. Various experimental observations have led to the following interpretation of these reactions. Reaction 1 corresponds to the binding of substrate yielding the initial Michaelis complex. Reactions 2 and 3 are two pH-independent reactions, ascribed to substrate-induced changes in the positions of active-site groups. Reaction 4 is a pH-dependent reaction (pK = 5.9) which involves the imidazole of His-57 but which is not the formation of a tetrahedral intermediate, oxazolinone, or acyl enzyme. The slowest detected step corresponded to the acylation reaction. No evidence for the accumulation of a tetrahedral intermediate was obtained. Spectral, kinetic, and thermodynamic data for these reactions are presented, as is justification for the relevance of these findings to the reaction under physiological conditions. These results demonstrate the utility of subzero temperatures in enzyme mechanism studies, especially with regard to allowing the accumulation of intermediates which may be quite stable at appropriate values of pH and low temperature.

Although chymotrypsin-catalyzed reactions have been very extensively studied, relatively little information is available concerning the dynamic processes occurring during the catalysis. The currently accepted pathway for the reaction involves a covalent acyl enzyme intermediate, EA in (1), in addition to the initial noncovalent Michaelis complex, ES (Bender and Kilheffer, 1973; Fastrez and Fersht, 1973b).

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} EA \xrightarrow{k_3} E + P_2 \qquad (1)$$
$$\qquad\qquad\qquad\quad + \qquad\qquad\quad P_1$$

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