Some Effects of Adenylylation on the Biosynthetic Properties of the Glutamine Synthetase from *Escherichia coli**

A. Ginsburg, J. Yeh, S. B. Hennig, and M. D. Denton†

ABSTRACT: The variation of Mg^{2+}- and Mn^{2+}-dependent biosynthetic activities as a function of the average extent of adenylylation ($\bar{n}$) of glutamine synthetase has been studied. The *in vitro* adenylylation of glutamine synthetase, which provided additional enzyme forms for study (together with a method for partially purifying the adenosine triphosphate–glutamine synthetase adenylyltransferase of *Escherichia coli*), are described also. Using spectrophotometric Mg^{2+} and Mn^{2+} assay systems for measuring initial velocities under saturating conditions and only highly purified glutamine synthetase forms ($\bar{n}$ = 0.8–12), sigmoid curves of specific activity *vs.* $\bar{n}$ were obtained. The addition or removal of 5′-adenylyl groups at either extreme of adenylylation ($\bar{n}$ = 0.8–3, $\bar{n}$ = 8.5–12) produces the most profound change in the Mg^{2+}- or Mn^{2+}-dependent activities. At intermediate stages of adenylylation ($\bar{n}$ = 3–8), the expressions of Mg^{2+} and Mn^{2+} activities are less affected by the covalent attachment of 5′-adenylyl groups to glutamine synthetase. With optimal assay conditions, unadenylylated subunits appear to be specifically activated by Mg^{2+} (pH optimum 7.5–7.6), whereas adenylylated subunits have an absolute requirement for Mn^{2+} (pH optimum 6.5–6.6) as the activating divalent cation. This conclusion, which is based on activity extrapolations to n = 0, and kinetic results obtained when n = 12, corroborates a previous report. Therefore, heterologous subunit interactions between adenylylated and unadenylylated subunits in the dodecameric glutamine synthetase molecule appear to influence V_{max}, as well as K'_m. In addition, unadenylylated, Mn^{2+}-activated glutamine synthetase forms express significant activity with D-glutamate which is decreased by adenylylation. With the natural substrate, L-glutamate, present, D-glutamate is about equally effective in inhibiting the L-glutamate activity of both Mn^{2+}-activated unadenylylated and adenylylated forms of glutamine synthetase. The formation of an active complex with D-glutamate, adenosine triphosphate, and ammonia appears to be related to a unique conformation that adenylylated subunits can assume in a molecule containing few 5′-adenylyl groups. The binding of L-[^{14}C]glutamate at pH 7.3 (4°) to a preparation of glutamine synthetase containing 0.8 equiv of 5′-adenylyl groups per mole of enzyme was measured. Although at 10^{-3} M L-glutamate, 12 equiv of L-glutamate was bound per mole of enzyme, the binding curve suggests that the maximum number of enzyme sites for L-glutamate is $\gg$12, with an apparent association constant of $\sim 10^3$ mole^{-1}. Parallel kinetic studies with the same enzyme preparation show high- and low-affinity sites for L-glutamate, with $K'_m \simeq 2.6 \times 10^{-5}$ and 2×10^{-4} M, respectively. There is no obvious correlation between the binding and kinetic results with L-glutamate, and this is discussed with the presentation of a scheme for the interaction of substrates with glutamine synthetase.

For pertinent background references to these studies, please refer to the introduction of the accompanying paper (Denton and Ginsburg, 1970).

The interpretation of the combined results from direct binding and kinetic studies with the glutamine synthetase from *Escherichia coli* depends largely upon a knowledge of the absolute divalent cation specificity of adenylylated and unadenylylated subunits in the biosynthetic reaction. The previous results of Kingdon *et al.* (1967) showed that adenylylation of glutamine synthetase linearly changed the divalent cation requirement from Mg^{2+} to Mn^{2+} in a colorimetric assay of the biosynthetic reaction (Woolfolk *et al.*, 1966). The extrapolations of these data (Stadtman *et al.*, 1968) indicated that the unadenylylated enzyme would have no activity in a Mn^{2+}-activated assay, and, conversely, that the fully adenylylated enzyme would be inactive in a Mg^{2+}-activated biosynthetic assay. The studies presented here were initiated to learn whether or not adenylylation can influence the maximum activity expressed by each type of subunit. Since it became apparent that the activity of each type of subunit is *not* expressed independently of the other in either a Mn^{2+}- or Mg^{2+}-dependent biosynthetic assay, the question of the absolute divalent cation specificity of adenylylated or unadenylylated subunits had to be reexamined. The sensitive spectrophotometric assay method of Kingdon *et al.* (1968) was used in these studies to measure initial velocities and to determine optimal assay conditions. Isolated glutamine synthetase preparations from *E. coli* (grown under conditions to yield preparation I or II (Shapiro *et al.*, 1967)), as well as enzyme preparations obtained by *in vitro* adenylylation of glutamine synthetase (Kingdon *et al.*, 1967; Wulff *et al.*, 1967; Ginsburg, 1970), were purified to homogeneity by the procedure of Woolfolk *et al.* (1966) for the activity measurements. The purified glutamine synthetase preparations studied had 0.8–12 equiv of covalently bound 5′-adenylyl groups per mole (600,000 g) of enzyme.

Equilibrium binding and kinetic studies of the interaction of glutamine synthetase with L-glutamate are reported also.

Experimental Section

Materials. The sodium salts of ATP, L-glutamate, L-glutamine, DPNH, Tris, and crystalline RNase A were obtained

* From the Laboratory of Biochemistry, Section on Enzymes, National Heart Institute, National Institutes of Health, Bethesda, Maryland. *Received September 23, 1969.*

† Present address: Thorndike Memorial Laboratory, Boston City Hospital, Boston, Massachusetts.

from the Sigma Chemical Corp., and of phosphoenolpyruvate from Boehringer Mannheim Corp. The latter also supplied crystalline ammonium sulfate suspensions of lactic dehydrogenase and pyruvate kinase (essentially free of myokinase activity). Crystalline DNase was obtained from Worthington Biochem. Corp. Uniformly labeled L-[^{14}C]glutamic acid, [^{14}C]ATP, and [α-^{32}P]ATP were obtained from Amersham Searle Corp., New England Nuclear Corp., and International Chemical and Nuclear Corp., respectively. Imidazole and maleic acid were obtained from Eastman Organic Chemicals; stock imidazole solutions were treated lightly with charcoal and filtered to remove any yellow color; maleic acid, recrystallized from acetone, was a gift from Dr. L. Tsai. Sephadex (G-50, fine) was obtained from Pharmacia. Non- and isotopically labeled compounds were determined to be >97% pure by the techniques used previously (Ginsburg, 1969).

Deionized water with a conductivity $\leq 1.7 \times 10^{-6}$ ohm^{-1} was obtained from a water deionizing unit (Model DJ-128) of the Crystal Research Laboratory, Inc., and this water was used exclusively for the preparation of solutions. Enzyme grade ammonium sulfate was obtained from Mann Research Laboratory, Inc.

Glutamine synthetase as isolated from *E. coli* by the procedure of Woolfolk *et al.* (1966) contains varying equivalents of 5′-adenylyl groups covalently bound to the protein (Shapiro *et al.*, 1967). The enzymes designated as $E_{\overline{0.8}}$, $E_{\overline{1.2}}$, $E_{\overline{2.3}}$, $E_{\overline{9}}$, $E_{\overline{10.4}}$, and $E_{\overline{11.2}}$[1] were isolated directly from *E. coli* after growth under different conditions (Kingdon and Stadtman, 1967), and these were generous gifts from Drs. J. Ciardi, F. Cimino, W. Anderson, H. K. Kingdon, and B. M. Shapiro of this laboratory. All enzymes show >95% homogeneity by disc gel electrophoresis and ultracentrifugation; we were fortunate in having more than one preparation of each $E_{\overline{0.8}}$, $E_{\overline{1.2}}$, and $E_{\overline{11.2}}$, plus some other preparations at either extreme of adenylylation with $\bar{n}$ only estimated, for activity assays. Only the most active enzymes of certain 5′-adenylyl content (see below) are reported in the measurements of absolute specific activities. The preparations of $E_{\overline{1.2}}$, $E_{\overline{9}}$ (Shapiro *et al.*, 1967), and $E_{\overline{2.3}}$ (Denton and Ginsburg, 1969) have been described elsewhere.

Methods

Instrumentation. Radioactive determinations were by scintillation counting using the solution of Bray (1960) and the Nuclear-Chicago Mark I scintillation counter. If necessary, appropriate quench corrections from two-channel counting were applied to the data. The same aqueous volume was used for radioactive standards and samples to be counted. A Model PHM 25 radiometer equipped with as cale expander and Leeds and Northrup No. 124138 microelectrode assembly was used for pH determinations. A Cary 15 recording spectrophotometer, equipped with a thermostable cell compartment, was used for spectral determinations and enzyme assays at 25.0°. A Unicam (Model SP 800) spectrophotometer, equipped with four-cell thermostatable brass blocks (sample and reference cell holders with contact pins between cells and the block), a SP 825 program controller, and a SP 830 autocell changer, was used for the enzyme assays at 20.0 and 37.0°. The control and monitoring of the temperature was as previously described (Ginsburg and Carroll, 1965). Assay mixtures were equilibrated in the cell block at the desired temperature for 5–10 min before initiating the reaction (see the assay methods below). Generally, four reaction rates were measured simultaneously over a 20-min period.

Preparation of 5′-[^{14}C]Adenylyl Glutamine Synthetase. In vitro adenylylation of the $E_{\overline{2.3}}$ preparation was performed with [^{14}C]ATP and varying amounts of the ATP–glutamine synthetase adenylyltransferase in order to obtain enzymes of known intermediate states of adenylylation.[2] In reaction volumes of 0.3 ml, the final concentrations were 5 mM [^{14}C]ATP ($\sim 10^6$ cpm/μmole), 2 mM L-glutamine, 10 mM Tris at pH 7.6, 50 mM $MgCl_2$, and in the five separate experiments of Table I (1) 0, (2) 0.45, (3) 0.90, (4) 1.87, or (5) 3.75 mg per ml concentrations were used of an approximately 20-fold purified fraction (see below) of the ATP–glutamine synthetase adenylyltransferase (1 mg catalyzing the attachment of ~220 mμmoles of 5′-adenylyl groups to glutamine synthetase in 15 min at 37°), and 23.3 mg/ml of $E_{\overline{2.3}}$ (exhaustively dialyzed beforehand against 0.01 M $MgCl_2$ and 0.02 M imidazole at pH 7.5 (4°) to displace Mn^{2+} from the $E_{\overline{2.3}}$ preparation). The reaction components *without* $E_{\overline{2.3}}$ were first combined and incubated for 5 min at room temperature, under which conditions a stable [^{14}C]ATP–adenylyltransferase complex is formed. Then, 7 mg of $E_{\overline{2.3}}$ was added and the reaction mixture was transferred to 37° for 1 min, and then iced and immediately gel-filtered at 4° through a 0.9 × 60 cm column of G-50 Sephadex, using an elution buffer at pH 7.5 of 0.01 M Tris, 50 mM $MgCl_2$, and 1 mM glutamine. The absorbancies at 280 and 260 mμ of the effluent fractions (1 ml) were monitored. Fractions 12 and 13 (containing ~65% of the total glutamine synthetase) were combined and 0.1-ml aliquots were taken for direct scintillation counting, and for the measurement of heat-stable, trichloroacetic acid precipitable radioactivity as follows: 2 ml of 7% trichloroacetic acid at room temperature and pH ~1 (prepared daily from a 50% trichloroacetic acid stock solution) is added to the aliquot of radioactive protein. (If <0.1-ml aliquots of a more concentrated radioactive protein solution (4–30 mg/ml) are taken, it is necessary to first dilute the protein into 1 ml of 0.01 M Tris buffer at pH 7.6 before adding 2 ml of 7% trichloroacetic acid. This dilution prevents the precipitate formed by the trichloroacetic acid addition from adhering to the walls of the 13 × 100 mm Pyrex test tubes used, without affecting the quantitative precipitation of the radioactive glutamine synthetase by trichloroacetic acid.) After boiling for 15 min, the precipitate is collected by filtration through a 0.45 μ Type HA Millipore filter using suction, and is washed on the filter with 10–5-ml volumes of 7% trichloroacetic acid. After draining, the filters are dissolved in 20 ml of the solution of Bray (1960) and counted. The heat-

[1] Abbreviations used are: $E_{\bar{n}}$ represents glutamine synthetase (Gln Syn) preparations (E) adenylylated to an *average* extent $\bar{n}$, where $\bar{n}$ is expressed as the average number of 5′-adenylyl groups per enzyme molecule of mol. wt. 600,000. The molecular distribution of the 5′-adenylyl groups in a particular enzyme preparation is unknown, although at maximum adenylylation $\bar{n} = 12$ or one adenylyl group per enzyme subunit. The 5′-adenylyl group is covalently bound to the enzyme through a stable phosphodiester bond to a specific tyrosyl hydroxyl group of the polypeptide enzyme subunit structure (Shapiro and Stadtman, 1968b); PEP, phosphoenolpyruvate.

[2] Most of the procedures described developed directly from studies on the ATP: glutamine synthetase adenylyltransferase of *E. coli*.

TABLE I: Radioactive Adenylylated Glutamine Synthetase Preparations.

Expt	$\bar{n}$ (Before Repurification)[a]	$\bar{n}$ (After Repurification)[b]	$\bar{n}$ (Cor)[b]	A_{260}/A_{290}[c]	$\bar{n}$ (From A_{260}/A_{290})[c,d]	Changes in Biosynthetic Activities[e]: % with Mg^{2+} Activation	% with Mn^{2+} Activation
1	2.4	2.45	2.3	1.090	3.0	100	100
2	3.42	3.36	3.2	1.152	4.0	77	108
3	4.34	4.39	4.2	1.185	4.5	77	138
4	6.09	5.95	5.8	1.288	6.0	66	169
5	8.00	8.23	8.1	1.366	7.2	51	200
6	11.6	12		1.717	12.4	2	398
7	12.0[f]			1.621	11.0	1	419

[a] From the trichloroacetic acid assay (see Methods):

$$\bar{n} = \bar{n}^0 + \bar{n}^* = (12)_{max} = \bar{n}^0 + \frac{\text{trichloroacetic acid insoluble cpm}}{\text{cpm of [}^{14}\text{C]ATP/}\mu\text{mole of ATP}} \times \frac{600{,}000}{\mu\text{g of Gln Syn added to trichloroacetic acid}}$$

where $\bar{n}$ is the average extent of adenylylation of the glutamine synthetase (Gln Syn) preparation with $\bar{n}^0$ equal to an average of 2.3 equiv of 5′-adenylyl groups/mole of the unlabeled Gln Syn used as starting material and $\bar{n}^*$ equal to the equiv of 5′-[^{14}C]-adenylyl groups incorporated per mole of Gln Syn in the adenylylation reaction. [b] As in footnote *a*, $\bar{n} = \bar{n}^0 + \bar{n}^*$ where $\bar{n}^*$ is measured by directly determining the radioactivity of the repurified Gln Syn. The value of $\bar{n}^*$ from expt 1 represents a blank for noncovalently bound radioactivity of $E_{\overline{2.3}}$, and was used to correct the $\bar{n}$ values (column 3) of expt 2–5. [c] All absorbancy values are approximately for corrected for any light scattering by assuming that Gln Syn does not absorb at 340 mμ, and multiplying the apparent absorbance of the enzyme so lution at 340 mμ by the ratio: $\lambda^4{}_{340\ m\mu}/\lambda^4{}_{m\mu}$ (Schauenstein and Bayzer, 1955). This calculated value is substracted from the absorbancy determined at λ, and is minimized by clarifying protein solutions by centrifugation before spectral determinations so that $A_{340\ m\mu} < 0.02$. Protein concentrations ≃1 mg/ml of 0.02 M imidazole-chloride, 0.10 M KCl, and 0.001 M $MnCl_2$ (pH 7.1). [d] $\bar{n} \simeq 15.00\ (A_{260\ m\mu}/A_{290\ m\mu}) - 13.31$ (from Shapiro and Stadtman (1970)) based on values at neutral pH for 10^{-6} M E_0 of $A_{290\ m\mu} = 0.231$ and $A_{260\ m\mu} = 0.205$, and for 10^{-6} M protein-bound AMP having the molar absorbance of free AMP ($A_{260\ m\mu} = 0.0153$ (Boch *et al.*, 1956)). This derived expression assumes that the covalently bound 5′-adenylyl groups do not perturb the absorbancy of the protein at 290 mμ while contributing linearly to the absorbancy at 260 mμ in an amount equivalent to the same concentration of free AMP. [e] See Table II (Methods) and Figure 2a,b (Results). No significant differences were obtained in assays before and after repurification of the $E_{\bar{n}}$ samples. [f] $E_{\overline{2.3}}$ adenylylated with [α-^{32}P]ATP instead of [^{14}C]ATP (see Methods).

stable, trichloroacetic acid precipitable radioactivity (see Table I) gives a direct measure of the amount of 5′-[^{14}C]-adenylyl groups incorporated into glutamine synthetase, when combined with the specific radioactivity of [^{14}C]ATP used. This latter value was conveniently determined from the [^{14}C]ATP fractions off the Sephadex G-50 column. The small difference between the total and trichloroacetic acid precipitated, heat-stable radioactivity is due to the amount of [^{14}C]-ATP noncovalently complexed under these conditions to the adenylyltransferase and the glutamine synthetase (<0.5 mμmole of [^{14}C]ATP/mg of total protein).

For preparing E_{12} of specific radioactivity of ~10^7 cpm/μmole of E_{12}, 3.46 mg of the adenylyltransferase preparation was preloaded at 25° with either [^{14}C]ATP or [α-^{32}P]ATP as described above, and then 5 mg of $E_{\overline{2.3}}$ was added to a final volume of 0.3 ml (expt 6 or 7, respectively, of Table I).[8] The reaction mixtures were incubated at 37° for 30m in, gel filtered at 4° (as above), and the protein fractions (containing ~95% of the glutamine synthetase) were pooled. In the pooled protein fractions, ~50% of the total radioactivity was equal to the amount of radioactive isotope covalently bound to the E_{12} preparations.

Repurification of the Radioactive, Adenylylated Glutamine Synthetase Preparations. The acetone and acid ammonium sulfate steps of the glutamine synthetase purification procedure of Woolfolk *et al.* (1966) were used. Slight modifications were introduced to compensate for the low protein concentrations of the adenylylated enzyme fractions. The following repurification procedure removes the adenylyltransferase, and the proteins introduced with this activity, and >97% of the noncovalently, protein-bound radioactivity. (All steps are at 4° and centrifugations are at 11,000–17,000 rpm for 15 min in a Sorvall centrifuge unless otherwise noted.)

Step 1. Saturated ammonium sulfate (30% by volume) and $MnCl_2$ to a final concentration of ~0.01 M were added to each adenylylated glutamine synthetase preparation. After equilibration, the pH was carefully adjusted to pH ~4.45 (±0.05 pH unit) with 1 M acetic acid. The precipitate was

[8] In large-scale adenylylation reactions, 10–15 units of the adenylyltransferase/mg of unadenylylated glutamine synthetase ($E_{\overline{0.8}-\overline{3}}$) has been found to yield E_{12} after about 45 min at 37°.

collected immediately by centrifugation, suspended in the 0.01 M imidazole–0.01 M $MnCl_2$ (pH 7.5) buffer used throughout the purification procedure, and adjusted to pH 7 with 1 M NH_4OH. (Best yields are obtained by allowing some time, even overnight, for redissolving the precipitate at pH 7.) This solution is clarified by centrifugation.

Step 2. Acetone at room temperature was added to a final volume of 46% to the supernatant from step 1, and the solution stirred for 5 min and then cooled in ice for 15 min. After collecting the precipitate by centrifugation, it was suspended immediately in the 0.01 M imidazole–0.01 M $MnCl_2$ buffer and adjusted to pH 7 with 1 M NH_4OH. This solution was clarified by centrifugation.

Step 3. Cold saturated ammonium sulfate (20–30% by volume) was added to the supernatant from step 2, and the acid precipitation procedure of step 1 was repeated.

Step 4. Step 3 was repeated and each glutamine synthetase preparation ($E_{\bar{n}}$) was dialyzed against 600–1000 volumes of 0.02 M imidazole-chloride, 0.10 M KCl, and 0.001 M $MnCl_2$ buffer (pH 7.5 at 4°; pH 7.1 at 25°) with several buffer changes. The dialyzed enzyme solutions were clarified and stored at 4°. Some of the properties of these samples are given in Table I.

The extent of adenylylation of native glutamine synthetase preparations ($\bar{n}$) was estimated by spectral (Shapiro and Stadtman, 1970; Table I) and γ-glutamyl transfer assay (Stadtman *et al.*, 1968) methods. Either method has an approximate accuracy of $\bar{n} \pm 1$. Usually, a number of both the spectral and assay analyses were averaged for each purified enzyme preparation in order to assign a value of $\bar{n}$ with some certainty (±0.4). In practice, the assay method was found to be more reliable than the spectral technique. A purified enzyme is required for spectral analysis, and any perturbation of the absorbancy at 290 or 260 mμ will affect the calculation of $\bar{n}$ (see footnote *d*, Table I). The γ-glutamyl transfer assay method may be used with purified or inhomogeneous glutamine synthetase fractions, since this method is based on an inhibition of this activity of adenylylated subunits by high concentrations of $MgCl_2$ when present in the Mn^{2+}-dependent assay system at pH 7.15 which, in the absence of $MgCl_2$, measures equivalent activities of both adenylylated and unadenylylated subunits (Stadtman *et al.*, 1968). For 5′-[^{14}C]adenylyl glutamine synthetase, the trichloroacetic acid assay (described above) specifically gives the number of 5′-[^{14}C]adenylyl groups (of known specific radioactivity) incorporated into glutamine synthetase (see footnote *a*, Table I). During the removal of radioactive 5′-adenylyl groups from the enzyme by the action of snake venom phosphodiesterase (Shapiro *et al.*, 1967; Stadtman *et al.*, 1968) or by deadenylylating activity (Shapiro and Stadtman, 1968a), the fraction of perchloric acid soluble radioactivity multiplied by the initial $\bar{n}$ gives a measure of $\bar{n}$ after deadenylylation. (This calculation assumes that the radioactive and nonradioactive 5′-adenylyl groups are released at the same rate; *i.e.*, that there is nothing unique about the 5′-adenylyl groups originally bound to unadenylylated glutamine synthetase preparations.)

Spectral Constants; Protein Determinations. (All ultraviolet absorbancy measurements are made on enzyme solutions that have been clarified by centrifugation and are corrected for any light scattering, due to residual turbidity, by applying a fourth-power extrapolation of the absorbancy at 340 mμ as described in footnote *c* of Table I.)

Taut $E_{\bar{n}}$ Forms. For native glutamine synthetase preparations of unknown adenylylation state, a specific absorption constant of $A^{0.1\%}_{290\,m\mu,1cm} = 0.385$ (Shapiro and Stadtman, 1970) is used for the determination of enzyme concentration. If the extent of adenylylation has been estimated by other measurements (see above), an empirical fit to most glutamine synthetase preparations obtained in this laboratory yields: $A^{0.1\%}_{280\,m\mu,1cm} = 0.733 + 0.05\,(\bar{n}/12)$. If $\bar{n}$ is known, the specific absorption constant at 280 mμ is used in preference to that at 290 mμ for greater accuracy in the determination of enzyme concentration.

Relaxed $E_{\bar{n}}$ Forms. The reversible spectral perturbation (involving the exposure to solvent of 12–24 tyrosyl and 12–24 tryptophanyl residues per enzyme molecule) that is produced by the removal, or by chelation with excess EDTA, of specific divalent cations (Mn^{2+}, Mg^{2+}, or Ca^{2+}) from taut (active) glutamine synthetase has been described previously (Shapiro and Ginsburg, 1968). Using the same methods, the difference spectrum between *taut* (active) $E_{\overline{2.3}}$ and *relaxed* (inactive) $E_{\overline{2.3}}$ (to which excess EDTA to divalent cation was added) was the same as that obtained between the taut and relaxed forms of E_{12}. The taut–relaxed difference spectra for $E_{\overline{2.3}}$ and E_{12} have maxima at 290.3 ($\Delta\epsilon$ 1.73 × 10^4 M^{-1} cm^{-1}) and 283.8 mμ ($\Delta\epsilon$ 1.29 × 10^4 M^{-1} cm^{-1}), and minima at 286.4 ($\Delta\epsilon$ 1.05 × 10^4 M^{-1} cm^{-1}) and 295 and 263 mμ ($\Delta\epsilon$ 0). Since the taut–relaxed difference spectrum is apparently independent of $\bar{n}$, specific absorption constants for 1-mg/ml concentrations of relaxed $E_{\bar{n}}$ could be calculated to be: $A_{290\,m\mu,\,1\,cm} = 0.356$ and $A_{280\,m\mu,\,1\,cm} = 0.716 + 0.05\,(\bar{n}/12)$.

Glutamine Synthetase Assays. For uniformity, each preparation of $E_{\bar{n}}$ used in the activity determinations was diluted to a concentration of ~1 mg/ml with the dialysate (0.02 M imidazole-chloride, 0.10 M KCl, and 0.001 M $MnCl_2$, pH 7.1), enzyme concentration was determined (see above), and stored at 4°. Under these conditions the enzyme activities are stable for months. If further dilutions of the $E_{\bar{n}}$ preparations were required for the assays, these were made with the dialysates in ice as needed. The spectrophotometric assay which measures ADP formation by coupling with pyruvate kinase and lactate dehydrogenase activities (Kingdon *et al.*, 1968) was used. The saturating conditions for measuring the Mg^{2+}- and Mn^{2+}-dependent biosynthetic activities of $E_{\bar{n}}$ forms are given in Table II, and these were used unless otherwise noted. The conditions shown in Table II are optimal for all adenylylation states, with the exception of the L-glutamate concentration for the Mn^{2+}-activated glutamine synthetase at low adenylylation states (see Results section) which is slightly inhibitory. No inhibitory effects with high ammonia concentrations, or with 5 mM ATP, were observed. Reaction rates usually were linear until all the DPNH was oxidized (2–20 min), at which time more DPNH could be added to the cuvet. All initial reaction rates reported were proportional to enzyme concentration. Using initial velocities, the specific activity is expressed as the μmoles of ADP formed per min per of enzyme. For the L-glutamate saturation studies, the conditions of footnote *e* in Table II were used.

Equilibrium dialysis techniques were as previously described (Ginsburg and Mehler, 1966; Ginsburg, 1969). A stock solution of the $E_{\overline{0.8}}$ preparation (10 mg/ml) used for these studies was predialyzed at 4° against three different 1000 volumes of 0.02 M imidazole-chloride, 0.10 M KCl, and 0.001 M $MnCl_2$ (pH 6.9, 20°, pH 7.3, 4°). The last dialysate was saved and

used for protein dilutions and standard solutions of L-[^{14}C]-glutamate (10^5–10^6 dpm/μmole of L-glutamate). In the equilibrium binding studies, the total amount of L-[^{14}C]glutamate always was kept in excess of the concentration of enzyme binding sites by using appropriate dilutions of the stock $E_{\overline{0.8}}$ solution (0.2–6.6 mg/ml of $E_{\overline{0.8}}$). At the low concentrations, the larger Techni-Lab Model E-5 (5 ml) cells were used to increase the sample size removed for analysis. After equilibration (48–72 hr at 4°), suitable aliquots were removed simultaneously from the protein and solvent sides of the dialysis cells for radioactivity, protein concentration, enzyme activity, and pH determinations. The pH remained constant at pH 6.90 ± 0.02 units at 25°; the enzyme activity was stable also. Since L-glutamate is a substrate of glutamine synthetase, the L-[^{14}C]glutamate in the solvent after equilibrium dialysis experiments with this enzyme was examined by electrophoresis in 4% formic acid (Dreyer, 1960), followed by exposure of the paper on X-ray film and development with ninhydrin. The L-[^{14}C]glutamate was judged to be unchanged through contact with the enzyme since it migrated with the standard radioactive and nonradioactive L-glutamate.

As is usual, the radioactivities of the protein and solvent sides of the membrane (accounting for the amount of L[^{14}C]-glutamate added initially within 5%) were used to calculate the total (protein bound plus free) and the free concentrations, respectively, of L-[^{14}C]glutamate. Even though glutamine synthetase did not appear to be saturated with L-[^{14}C]glutamate at ~1 mM (see Results), the limitation of this method in accurately measuring small differences between large amounts of radioactivity, prevented measurements at >1 mM L-[^{14}C]glutamate. Throughout these studies, a molecular weight of 600,000 for the enzyme was used to calculate molar concentrations of protein from absorbancy measurements at 280 mμ (Shapiro and Ginsburg, 1968). The data were analyzed as described previously (Ginsburg, 1969).

Partial Purification of the ATP–Glutamine Synthetase Adenylyltransferase. The method outlined here is designed for obtaining (1) the adenylyltransferase as a by-product of the glutamine synthetase isolation procedure of Woolfolk *et al.* (1966), and (2) a 20–30-fold purified adenylyltransferase which is suitable for the adenylylation of purified glutamine synthetase (see Table III).

Step 1. The preparation of the cell extract from *E. coli* strain W cells grown on glycerol–glutamate and harvested in stationary growth phase (see Kingdon and Stadtman, 1967; Shapiro and Stadtman, 1970), and the subsequent streptomycin precipitation steps are as described in Woolfolk *et al.* (1966).

Step 2. (a) The streptomycin precipitate from step 1 is immediately suspended with a rapid mechanical stirring at 4° in 8 volumes/g of precipitate of 0.05 M potassium phosphate–3 mM $MgCl_2$ buffer at pH 7.6. After stirring 10–12 hr to obtain a homogeneous suspension, 30 μg of crystalline RNase A and 0.4 μg of DNase (crystalline) are added per g of suspended precipitate. The mixture is then incubated at 30° with stirring until the nucleic acid fraction has been digested (10–20 hr). The progress of the nuclease action is followed by comparing the 260-mμ absorbancies of water-soluble to acid-soluble material as follows: one 0.2-ml aliquot of the streptomycin slurry is added to 1 ml of water and another 0.2-ml aliquot is added to 1 ml of 10% perchloric acid. After centrifugation, the 260-mμ absorbancies of the supernatants are determined. When the acid-soluble 260-mμ reading is the same as that of the water-soluble material, the nuclease digest is centrifuged at 4° (60 min at 14,000*g*). The supernatant (clear light yellow) is collected and the precipitate is washed with about $^1/_{15}$th volume of the buffer used throughout: 0.02 M Tris-chloride with 1 mM β-mercaptoethanol at pH 7.6–7.8, prepared with deionized water.

TABLE II: Saturating Conditions for the Mg^{2+}- and Mn^{2+}-Dependent Spectrophotometric Assay.

Compound[a] (Order of Addition to 1-ml Cuvet)	Mg^{2+} Activation (Final pH 7.5–7.6) (ml)	Mn^{2+} Activation (Final pH 6.5–6.6) (ml)
Water	0.525[b]	0.390[b]
Buffer[c]	0.200	0.200
0.90 M KCl–0.50 M $MgCl_2$ mixture	0.100	
0.90 M KCl–0.060 M $MnCl_2$ mixture		0.100
1.0 M NH_4Cl, pH 7	0.050	0.100
1.0 M L-glutamate pH 7[d]	0.030	0.100
0.10 M ATP, pH 6.8	0.050	0.050
DPNH, 15 mg/ml[e]	0.015	0.020
0.10 M PEP, pH 5.8[f]	0.010	0.010
Coupling enzymes[f]	0.010	0.020
Glutamine synthetase (E_n)	0.010 (0.1–5 μg)[e]	0.010 (1–20 μg)[e]

[a] ATP, PEP, and DPNH solutions stored in the frozen state; L-glutamate, coupling enzymes, and glutamine synthetase are stored at 4°. [b] Water volume is adjusted according to the amounts of glutamine synthetase and other solutions so that the final assay volume is 1.00 ml. [c] Buffer may be a stock solution of 0.25 M maleate, 0.25 M imidazole, and 0.25 M Tris, adjusted to pH 7.7–7.8 or 6.52–6.6 for the Mg^{2+} or Mn^{2+} assay systems, respectively. Alternatively, a stock solution of 0.5 M imidazole (pH 7.7), may be used in the Mg^{2+} assay. [d] L-Glutamate is omitted for the determination of ATPase activity in the glutamine synthetase preparation. [e] The DPNH solution (0.005 ml) is added with ~30 μg of DPNH/ml of water in the reference compartment, if the 0–0.1 slide-wire is used with ~0.1 the specified concentration of glutamine synthetase, which varies according to the extent of adenylylation of the enzyme preparation (see Results). [f] The less stable PEP and coupling enzymes (stored at 4° as a solution of 10 mg of lactate dehydrogenase–2.5 mg of pyruvate kinase per ml of 0.10 M KCl) are added just 5–10 min prior to assay measurements; the contents of the cuvets then are mixed and the cuvets are placed in the spectrophotometer. When the absorbancy at 340 mμ is constant (1–3 min), glutamine synthetase is added and the rate of DPNH oxidation is followed.

TABLE III: Summary of the Partial Purification of Adenylyltransferase.

Protein Fraction	Total Protein (g)	Specific Activity[a] (units/mg)	Yield (%)
Untreated extract[b]	236	~12	100
Solubilized streptomycin ppt;[c] first pH 4.4 ppt	5.23	62	11
Second pH 4.4 ppt; stored at pH 7.6	1.56	240	14
Third pH 4.4 ppt		310	

[a] Units of activity are expressed as mμmoles of 5′-adenylyl groups incorporated into glutamine synthetase in 15 min at 37°. Protein is determined by either the biuret or 280–260-mμ absorbancy method (Layne, 1957). The assay mixture (50 μl) contains: 20 mM Tris-chloride (pH 8), 50 mM $MgCl_2$, 2 mM L-glutamine, 75. mM [^{14}C]ATP or [α-^{32}P]ATP ($6–10 \times 10^5$ cpm/μmole of ATP), 4.8-mg/ml concentration of unadenylylated glutamine synthetase ($\bar{n}$ = 0–3), and sufficient adenylyltransferase to incorporate 0.8–5 moles of radioactive 5′-adenylyl groups into 1 mole of glutamine synthetase. The reaction is initiated at 37° by the addition of the adenylyltransferase and terminated by the rapid successive addition of 1 ml of 0.01 M Tris buffer (pH 7.6) and 2 ml of 7% trichloroacetic acid. After placing the tubes in boiling water for 15 min, the contents are filtered and counted as described in the method for preparing adenylylated glutamine synthetase. Values are corrected for a blank in which either the adenylyltransferase or $MgCl_2$ is omitted from the reaction mixture. [b] As in the purification table for glutamine synthetase (Woolfolk *et al.*, 1966), with 1130 g of frozen glycerol-glutamate-grown cells yielding also 400 mg of glutamine synthetase $E_{\overline{11}}$ purified through step 7 to a specific activity of 125. [c] Streptomycin precipitate (252 g) from the glutamine synthetase preparation. The low recovery of the adenylyltransferase activity in this step is due to an inefficient precipitation of this enzyme in the 1% (v/v) streptomycin sulfate precipitation step of the glutamine synthetase preparation (Woolfolk *et al.*, 1966). This loss in the adenylyltransferase is acceptable because glutamine synthetase is obtained from the supernatant of the streptomycin precipitation step.

(b) To the combined supernatant and wash of step 2a, solid ammonium sulfate is added slowly with stirring to 0.5 saturation (29.1 g of $(NH_4)_2SO_4$/100 ml of solution). After equilibration (may be overnight), the pH is adjusted to 4.4 with 1 M acetic acid. Equilibrate at this pH (final pH 4.4–4.5), with slow stirring at 4° for several hours, and then collect the precipitate by centrifugation. The precipitate is suspended in about $^1/_{15}$th of the supernatant volume of step 2a with the Tris–β-mercaptoethanol buffer, and the pH is adjusted to pH 7.6 with 1 N NH_4OH. (From this stage of the purification, care is taken to avoid metal contamination.) After slow stirring overnight, the enzyme suspension is clarified by centrifugation.

Step 3. To the supernatant solution from step 2b, 10% by volume of saturated ammonium sulfate (4°) is added, and the pH is adjusted to pH 4.4 with 1 M acetic acid. After equilibration at this pH (may be overnight), the precipitate is collected by centrifugation and suspended in the Tris–β-mercaptoethanol buffer, and the pH is adjusted to pH 7.6 with 1 N NH_4OH. (The volume should be $^1/_6$th to $^1/_{10}$th that of the previous fraction.) Equilibrate at pH 7.6 and then recentrifuge, discarding any precipitate. The adenylyltransferase in this opalescent, white supernatant fraction (protein concentration at about 25 mg/ml; $A_{280\ m\mu}/A_{260\ m\mu}$ = 1.4–1.6) is stored at 4°, under which conditions it is stable for months.

Step 4. To a portion of the solution from step 3, 10% by volume of ammonium sulfate is added, and the pH is adjusted slowly to pH 5.8 with 1 M acetic acid; centrifuge and discard the precipitate. The pH of the supernatant is adjusted to pH 4.4, and, after equilibrating at this pH for 30 min, the precipitate is collected by centrifugation and treated as in step 3. This step should give a further 1.3–2-fold purification (Table III with about 70% recovery of enzyme units ($A_{280\ m\mu}/A_{260\ m\mu}$ = 1.8.)

Results

Specific Activity Measurements. Figure 1 shows representative pH–activity profilesf or enzymes adenylylated to different extents. The pH of each assay was determined immediately after the velocity measurement at ~26° (see Methods, Table II). The Mg^{2+}-dependent activity, shown by the upper curves of Figure 1, has a pH optimum at pH 7.5–7.6. With increasing adenylylation, the pH–activity curves have less sharp maxima. Since Mg^{2+} activates only unadenylylated subunits in the biosynthetic reaction (see below), the difference in shape among the pH–activity profiles of different enzyme preparations must reflect some sort of subunit interaction. It cannot be said with certainty that the ionization of amino acid residues at the catalytic sites of unadenylylated subunits are influenced by heterologous subunit interactions because other complicated factors are involved in the expression of activity. The Mn^{2+}-dependent biosynthetic activity, which measures adenylylated subunits, is optimal at pH 6.5–6.6. At lower pH values than that indicated by the arrow (lower part of Figure 1), inactivation of the different enzyme forms was indicated by the velocity decreasing with time that the enzyme was exposed to the more acidic assay mixtures. This can account for the nonsymmetrical Mn^{2+}-activated pH curves. The pH dependence of the reaction of Mn^{2+}-activated $E_{\overline{2.3}}$ with D-glutamate instead of with the natural substrate, L-glutamate, is shown also. The pH optimum is more acidic with D-glutamate as the substrate, and no increase in this activity was observed in the higher pH range. Although the activity of $E_{\overline{2.3}}$ against D-glutamate is negligible in the Mg^{2+} assay at pH 7.6 and 25° (see Figure 3 below), it was possible that the activity with D-glutamate was being expressed by a Mn^{2+} activation of unadenylylated subunits. In the γ-glutamyl transfer assay with Mn^{2+} used as the activating divalent cation, the pH optimum of unadenylylated subunits is ~pH 8 (Stadtman *et al.*, 1968). However, the pH–activity profiles of Mn^{2+}-activated $E_{\overline{2.3}}$ with either L- or D-glutamate suggest that adenylylated subunits are involved in catalysis in both cases.

Figure 2a,b shows the Mg^{2+}- and the Mn^{2+}-dependent specific activities, respectively, of some different enzyme forms.

The points represent averages of multiple determinations with each enzyme preparation. Saturating substrate concentrations and the pH optima of different biosynthetic assay systems were used. Temperature was controlled at 20, 25, or 37°. The half-filled circles at 25° indicate the $E_{\bar{n}}$ samples derived from the $E_{\overline{2,3}}$ preparation. The solid lines of Figure 2a,b show that neither the Mg^{2+}- nor the Mn^{2+}-activated biosynthetic activities are linear functions of $\bar{n}$. This result conflicts with that obtained by Kingdon (see Stadtman *et al.*, 1968) and will be discussed below.

The open squares of Figure 2a show activity measurements on different mixtures of $E_{\overline{0,8}}$ and E_{12} which were iced for 1 hr prior to assay, and these activities are plotted according to the average extents of adenylylation of the mixtures. The linear response (dashed line) suggests that hybrids of intermediate adenylylation states were not formed at 4° in the mixtures of unadenylylated and adenylylated glutamine synthetase. The mixtures also had additive activities in the Mn^{2+} assay system but these results are not shown in Figure 2b in order to simplify this figure. It has been observed too that the successive addition of different enzyme forms directly to the assay cuvets in either the Mg^{2+} or Mn^{2+} assay systems gives additive rates of catalysis. Thus, there is no evidence that glutamine synthetase forms dissociate and reassociate at very low enzyme concentrations.

The dotted-dashed line of Figure 2a shows results obtained during the adenylylation of $E_{\overline{0,8}}$, using the phosphate biosynthetic assay of Woolfolk *et al.* (1966). As in the solid curves for the various native and adenylylated preparations, there is a dramatic decrease in activity between $\bar{n} \simeq 1$ and 3. Glutamine synthetase containing zero 5′-adenylyl groups has not been obtained so that the highest Mg^{2+}-activated specific activity cannot be measured. However, E_{12} has no Mg^{2+}-dependent activity so that the more rapid decrease in activity between $\bar{n} \simeq 8$ and 12 at the other extreme of adenylylation indicates that the Mg^{2+} activity is most affected by attaching 5′-adenylyl groups to the enzyme at either extreme of adenylylation. At intermediate stages, $\bar{n} \simeq 3$–8, adenylylation produces less of a decrease in the Mg^{2+}-dependent activity.

The Mn^{2+}-activated curves of Figure 2b show a converse relationship to those in Figure 2a. In this case, the inability to obtain E_0 makes the extrapolation to zero for this enzyme form tentative, as indicated by the dashed lines in Figure 2b between $\bar{n} \simeq 0$ and 0.8. The more marked increase in the Mn^{2+}-dependent activity at $\bar{n} \simeq 8$–12, and reasoning that there should be a similarity between the symmetry of the Mg^{2+} and Mn^{2+} activation curves, enabled us to conclude that the curves in Figure 2b probably are correctly extrapolated to zero for E_0.

Although the different temperatures did not seem to influence the curve shapes in Figure 2a,b, Arrhenius plots of the data revealed a discontinuity in the 1/T *vs.* log specific activity plots. In all cases, a lower apparent activation energy is observed at 37° than at 20–27°. Not enough data were obtained to determine the critical temperature. Dixon and Webb (1964) have pointed out that this type of discontinuity can arise if there are two successive reactions with different temperature coefficients so that the reaction with the higher temperature coefficient tends to become more rapid of the two at the higher temperature and the overall process is limited by the reaction with the lower temperature coefficient. Since glutamine synthetase reacts with three substrates in the biosynthetic reaction, this is a possible explanation. Alternatively, temperature-dependent conformations of the enzyme could be involved. It is of interest that L-glutamate, which is slightly inhibitory at 25° in the assays of the Mn^{2+}-activated unadenylylated forms (see Figure 4a), is less so at 37° so that the Arrhenius plots appear to approach linearity in these cases.

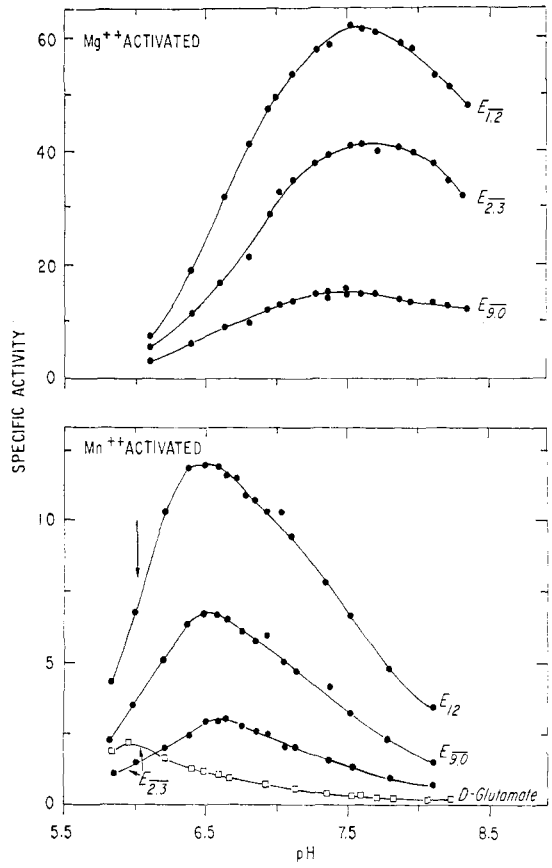

FIGURE 1: Activity–pH profiles for representative glutamine synthetase preparations ($E_{\bar{n}}$)[1] at ~26°. The upper and lower portions of the figure show Mg^{2+}- and Mn^{2+}-dependent specific activities, respectively, using the biosynthetic assay conditions of Table II (Methods) with mixed 0.25 M maleate, 0.25 M imidazole, and 0.25 M Tris buffers adjusted to different pH values. The pH values on the ordinate are from measurements made on the assay mixtures immediately following each activity determination. The pH–activity profile of the Mn^{2+}-activated $E_{\overline{2,3}}$ preparation, with D-glutamate instead of the natural L-glutamate substrate (□) is shown also.

Despite the fact that the glutamine synthetase of *E. coli* is extremely stable, there exists the possibility that inadvertent denaturation, or inactivation, occurred during enzyme purification which is reflected in the specific activity measurements. The repurification of the preparations that were adenylylated *in vitro* produced no significant changes in these activities (Table I). However, the sharpest decrease in the Mg^{2+}-dependent activity occurs between $\bar{n} = 1$ and 3 (Figure 2a). Since most enzyme forms were derived from $E_{\overline{2,3}}$, it was important to establish that there was not something unusual about the activity of the $E_{\overline{2,3}}$ preparation. Previous studies have established that most of the physical properties and the amino acid composition of the $E_{\overline{2,3}}$ preparation are the same as those of the $E_{\overline{1,2}}$ and E_9 enzyme forms (Shapiro and Ginsburg, 1968; Shapiro *et al.*, 1967; Denton and Ginsburg, 1969). In separate

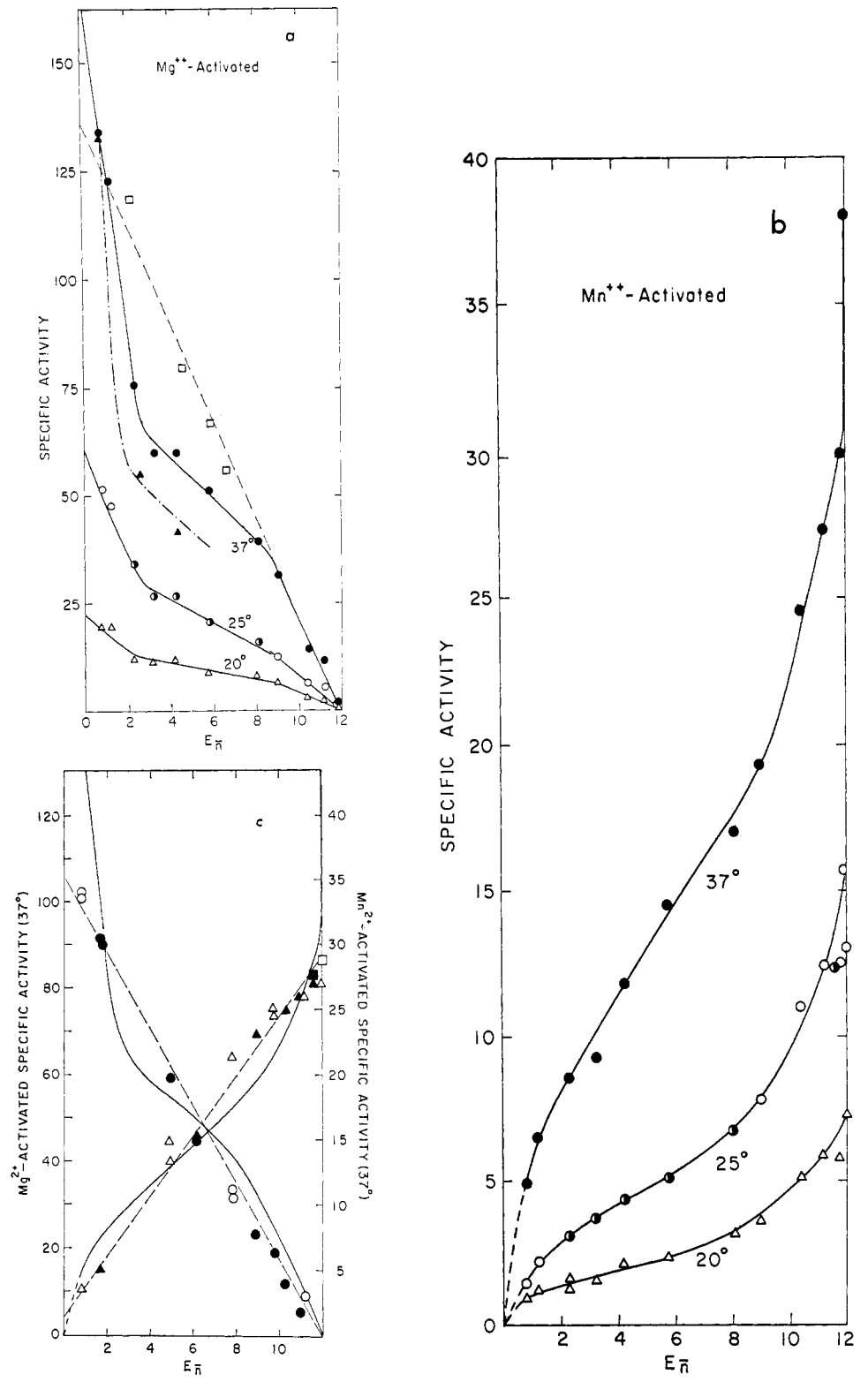

FIGURE 2: The variation of Mg^{2+}- and Mn^{2+}-dependent specific activities as a function of the average extent of adenylylation ($\bar{n}$) of the enzyme preparation ($E_{\bar{n}}$). (a) Approximately optimal activities of different enzyme forms ($E_{\bar{n}}$) in Mg^{2+}-activated spectrophotometric assays at 37, 25, and 20° are shown by the points associated with the solid lines (see Methods, Table II). The symbol (◑) at 25° indicates the enzymes ($E_{\bar{n}}$) derived from the $E_{2.3}$ preparation (see text). Results obtained during the adenylylation of the $E_{0.6}$ preparation, using the biosynthetic phosphate assay of Woolfolk *et al.* (1966) are shown by (▲–▲). Results obtained with mixtures of $E_{0.6}$ and E_{12} to give $E_{\bar{n}}$ which were incubated at 0° for 1 hr prior to assay are shown by (□– – –□). (b) The nearly optimal activities of different enzyme forms ($E_{\bar{n}}$) in Mn^{2+}-activated spectrophotometric assays at 37, 25, and 20° are shown (see Methods, Table II). (c) The Mg^{2+}- (○, ●) and Mn^{2+}- (△, ▲) activated specific activities at 37° (Table II) of radioactive E_{12} preparations (□, ■) after treatment with snake venom phosphodiesterase[4] to release either 5′-[^{32}P]adenylyl (open symbols) or 5′-[^{14}C]adenylyl (closed symbols) groups. These samples were kindly furnished by Dr. E. R. Stadtman,[4] and the dashed lines are dawn to approximately fit the points. The solid lines are copied from a and b for the 37° Mg^{2+}- and Mn^{2+}-activated curves, respectively.

studies,[4] the radioactive preparations of E_{12} prepared by adenylylating $E_{\overline{2.3}}$ (see Methods) were treated with snake venom phosphodiesterase to hydrolyze off different amounts of 5′-[^{14}C]adenylyl or 5′-[^{32}P]adenylyl groups. The Mg^{2+}- and Mn^{2+}-dependent specific activities of these samples,[4] after reactivation in the saturated assay systems at 37°, are shown in Figure 2c. The solid curves are the same as those in Figure 2a,b for the Mg^{2+}- and Mn^{2+}-dependent activities at 37°, respectively. Although the points in Figure 2c deviate form the solid curves, the most important result is that the $E_{\overline{0.8}}$ and $E_{\overline{1.6}}$ samples (*i.e.*, with $\bar{n}$ <2.3) have specific activities that are not very different from those of the native preparations. Thus, the activity of the $E_{\overline{2.3}}$ preparation does not appear to be unique, but rather reflects the adenylylation state of the enzyme.

The greater deviation in the Mg^{2+}- and Mn^{2+}-dependent activities of the phosphodiesterase-treated samples at high adenylylation states is not understood. It is of interest that the experimental data of Figure 2c approximately are fit by the two dashed lines shown. By these criteria, the snake venom phosphodiesterase would appear to produce glutamine synthetase forms in which each subunit type acts as a catalytically independent unit, in contrast to the behavior of native or adenylylated enzyme forms. Possibly there is a real difference in the mechanisms of the adenylylating and deadenylylating systems of *E. coli* and that of the snake venom phosphodiesterase. A seemingly more trivial explanation could be that the phosphodiesterase preferentially removes the radioactively labeled 5′-adenylyl groups in the initial stages of digestion. This would introduce an error into the calculations of $\bar{n}$ (see Methods), making the calculated values lower than they actually are for $\bar{n} > 6 \leq 11$. The fact that the Mn^{2+}-independent activities for $\bar{n} \geq 6$ were lower than the solid curve of Fgure 2c when the corresponding Mn^{2+}-dependent activities were high, suggests the possibility of errors in these estimated $\bar{n}$ values. However, such an explanation implies that the configuration of the enzyme about the originally unlabeled 5′-adenylyl groups (2.3 equiv) differs from that about these groups attached *in vitro* in such a way that somehow protects them against initial hydrolysis. It is reasonable to expect a random removal of 5′-adenylyl groups by the phosphodiesterase unless certain AMP-tyrosyl sites are protected by the adenylylated glutamine synthetase structure. Finally, it should be mentioned that another variable in measuring the activities of the phosphodiesterase-treated samples is introduced by relying on complete reactivation of the different relaxed enzyme forms[4] in the 37° assay systems. However, the E_{12} preparations had the same activities as the controls which were treated and stored with EDTA (Figure 2c), and all velocities attained constant linearity within 1 min. The reactivation of relaxed glutamine

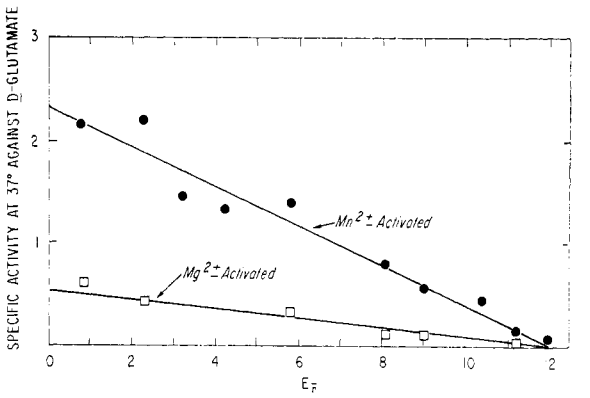

FIGURE 3: The ability of different enzyme forms ($E_{\overline{n}}$) to form ADP from D-glutamate, ATP, and ammonia in the Mg^{2+}- or Mn^{2+}-dependent biosynthetic assay systems at 37°. The assay conditions are as specified in Table II (Methods) except that D-glutamate was substituted for L-glutamate.

synthetase preparations in Mg^{2+}-activated assay mixtures has been previously reported (Kingdon *et al.*, 1968; Shapiro and Ginsburg, 1968), but these results do not guarantee that theoretical rates will be attained in all cases. It is hoped that future studies will elucidate the apparent discrepancies in the phosphodiesterase action.

Activity with D-*Glutamate.* Quite accidentally, it was discovered that the Mn^{2+}-activated $E_{\overline{2.3}}$ preparation catalyzed the formation of ADP from ATP, D-glutamate, and ammonia at significant rates. When this effect was investigated more thoroughly, it was found that the specific activities of different enzyme forms with D-glutamate varied as a function of adenylylation. These results are shown in Figure 3. The activities of the different enzyme forms with D-glutamate in the Mg^{2+}-dependent assay at pH 7.6 is <1–2% of the corresponding values with L-glutamate, and are therefore quite insignificant. However, the activity against D-glutamate in the Mg^{2+}-activated assay was not investigated as a function of pH. The D isomer does inhibit the Mg^{2+}-activated L-glutamate activity at pH 7.6. The decreasing catalytic activity of the Mn^{2+}-activated enzyme forms against D-glutamate with increasing adenylylation does not appear to be due to a decreased ability of adenylylated enzyme forms to bind the D isomer of the natural substrate. The results shown in Table IV indicate the D-glutamate is about equally effective in inhibiting the L-glutamate activity of either $E_{\overline{2.3}}$ or E_{12} when 1:1 mixtures of L- and D-glutamate were used in the saturating Mn^{2+} assay system. The pH optimum of the Mn^{2+}-activated $E_{\overline{2.3}}$ preparation with D-glutamate is more acidic than would be anticipated if Mn^{2+}-activated *unadenylylated* subunits were responsible for this activity (see above). If Mn^{2+}-activated unadenylylated subunits are not involved in the catalysis with D-glutamate, the Mn^{2+} activated *adenylylated* subunits in molecules containing few 5′-adenylyl groups must be in a favorable conformation for reacting with D-glutamate. The further adenylylation of these molecules could induce changes in the conformation of adenylylated subunit that make them unable to react with D-glutamate, but still able to combine with this substrate analog.

In the studies of Woolfolk *et al.* (1966) in which inorganic phosphate formation at 37° was measured, the biosynthetic reaction of the glutamine synthetase from *E. coli* with D-gluta-

[4] Independent studies were performed by Dr. Earl R. Stadtman in which the radioactive, adenylylated glutamine synthetase preparations (E_{12}) were digested with snake venom phosphodiesterase as described in Shapiro *et al.* (1967), and then stored in 0.01 M EDTA to stop the phosphodiesterase action. The varying amounts of 5′-[^{14}C]adenylyl or 5′-[^{32}P]adenylyl groups (0–11.2 equiv) hydrolyzed from E_{12} preparations were determined by the radioactivity released (see Methods). We are grateful to Dr. E. R. Stadtman for supplying us with samples of the phosphodiester digests for biosynthetic activity measurements. As a consequence of adding excess EDTA to divalent cation to inhibit the snake venom phosphodiesterase, the glutamine synthetase samples ($E_{\overline{n}}$) are in a relaxed state, but all reactivate rapidly (<1 min) in the assay mixtures of Table II (Methods) at 37°.

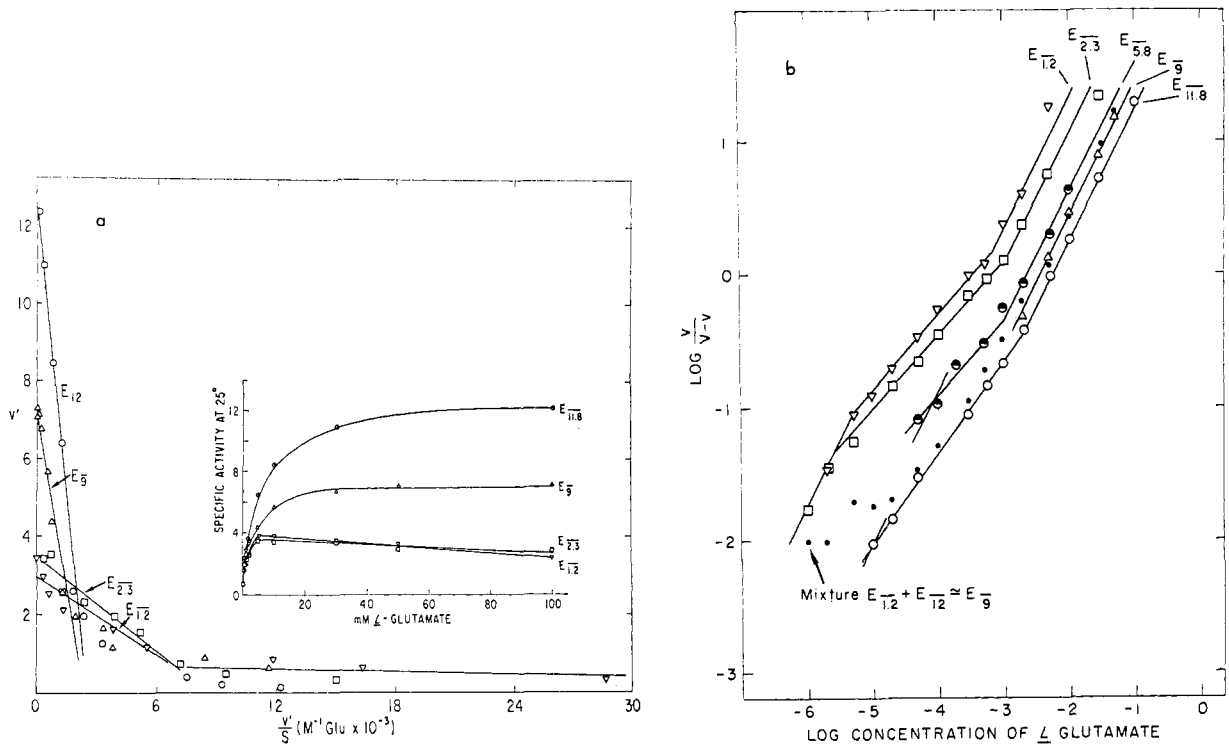

FIGURE 4: Kinetic L-glutamate saturation functions for different Mn^{2+}-activated enzyme forms. (a) L-Glutamate saturation curves in the Mn^{2+}-dependent assay at pH 6.5 and 25° for glutamine synthetase preparations ($E_{\overline{1.2}}$ (▽), $E_{\overline{2.3}}$ (□), $E_{\overline{9}}$ (△), $E_{\overline{11.8}}$ (◑), and E_{12} (○). The initial velocities (v') with varying L-glutamate concentrations (S) were measured as described in Methods (Table II). The v' *vs.* v'/S plot is analagous to that of Scatchard (1949) for binding measurements. (b) Hill plots of data obtained as in a for $E_{\overline{1.2}}$ (▽), $E_{\overline{2.3}}$ (□), $E_{\overline{5.8}}$ (◑), $E_{\overline{9}}$ (△), and $E_{\overline{11.8}}$ (○) and for a mixture of $E_{\overline{1.2}}$ and E_{12} (●) which has an average extent of adenylylation equivalent to that of $E_{\overline{9}}$; v is the initial velocity measured at each concentration of L-glutamate and V is the maximum velocity observed (see insert to a).

mate was only 4% the rate of that with L-glutamate. Presumably, Mg^{2+} was used as the activating divalent cation in these studies; the adenylylation state of the enzyme used then is unknown. The results of Figure 3 with the Mn^{2+}-activated unadenylylated glutamine synthetase from *E. coli* are similar to the observations of Levintow and Meister (1953) with the Mg^{2+}-activated mammalian glutamine synthetase. The mammalian enzyme utilizes both L- and D-glutamate with the rate of L-glutamine formation about three times more rapid than that of D-glutamine. The observations on the stereospecificity of the mammalian enzyme led Meister and coworkers to predict a stepwise mechanism for glutamine synthetase (see Discussion below), which was confirmed later by substrate binding studies (Krishnaswamy *et al.*, 1962).

TABLE IV: Some Catalytic Effects of D-Glutamate.[a]

Enzyme Preparation (mM)	$E_{\overline{2.3}}$ % Optimum Activity	E_{12} % Optimum Activity
L-Glutamate, 100	93	100
30	100	90
D-Glutamate, 100	33	1.5
30	29	
Mixture of 100 mM each of L- and D-glutamate	84	80
Mixture of 30 mM each of L- and D-glutamate	85	

[a] Mn^{2+}-dependent assay at pH 6.5 and 25° with either or both L- and D-glutamate and the usual other assay components at saturating concentrations (see Table I in Methods).

L-Glutamate Saturation Functions with Mn^{2+} Activation at pH 6.5. Figure 4a shows the kinetically determined saturation of various Mn^{2+}-activated enzyme forms with L-glutamate at pH 6.5 and 25°, with ATP and ammonia saturating for all enzyme forms (Table II and Methods). The plot of Figure 4a is analogous to that of Scatchard (1949) for binding data (Frieden, 1967), and is preferred for expanding the activity response at very low concentrations of L-glutamate. Observing that an increase in slope indicates a decrease in affinity, the different Mn–enzyme forms have high affinities for substrate at low L-glutamate concentrations that decrease as the concentration of L-glutamate is increased. The high-affinity data extrapolate to low apparent V'_{max} values, whereas large increases in velocity are associated with the low-affinity data. This type of negative interaction between Mn^{2+}-activated glutamine synthetase and L-glutamate is reported in the accompanying paper (Denton and Ginsburg, 1970) from results obtained in suboptimal Mn^{2+}-activated assays at pH 7.1. Although qualitatively similar results are obtained using either suboptimal or optimal Mn^{2+}-activated assay conditions, the results of Figure 4a are quantitatively different. The Mn^{2+}-activated enzyme forms

have increased affinities for L-glutamate and adenylylated forms are much more active under the more optimal assay conditions. Also, an inhibition of unadenylylated enzyme forms by high concentrations of L-glutamate is more apparent at pH 6.5 (see Figure 4a insert) than at pH 7.1. A greater inhibition by L-glutamate was observed with the $E_{1.2}^{-}$ preparation than with $E_{2.3}^{-}$. (Although not shown $E_{0.8}^{-}$ correspondingly was more sensitive to inhibition by L-glutamate than was the $E_{1.2}^{-}$ form.) The preparations E_9 and $E_{11.8}^{-}$ only are activated by increasing concentrations of L-glutamate since these enzyme forms are not saturated until L-glutamate is increased to ~100 mM.

Figure 4b illustrates Hill plots of the data from Figure 4a together with data obtained under the same conditions with the $E_{5.8}^{-}$ preparation and a mixture of $E_{1.2}^{-}$ and E_{12}. In contrast to the results obtained at pH 7.1 (Figure 6b in the accompanying paper, Denton and Ginsburg, 1970), only the data obtained with $E_{1.2}^{-}$ and $E_{2.3}^{-}$ at pH 6.5 approach a unit slope at the low L-glutamate concentrations. These data are obtained at the maximum sensitivity of the spectrophotometric assay system, but the high-affinity segment of the Hill plot for $E_{1.2}^{-}$ and $E_{2.3}^{-}$ can be extrapolated to zero on the ordinate to give an estimated K_m' value of $\sim 6 \times 10^{-5}$ M. The concentrations of L-glutamate required to produce half-saturation, or zero on the ordinate of Figure 4b, for the enzyme forms with $\bar{n}$ = 1.2, 2.3, 5.8, 9, and 11.8 were 0.35, 0.69, 2.3, 3.6, and 5.5 mM, respectively. All of the data have unit slopes at the high L-glutamate concentrations, and this indicates that finite interaction of the enzyme forms with high concentrations of L-glutamate occurs (Wyman, 1964, 1967). However, this is not true at the low L-glutamate concentrations with the adenylylated enzyme forms at pH 6.5. A pH decrease from 7.1 to 6.5 appears to more markedly increase the high and low affinities of the unadenylylated enzyme forms for L-glutamate. The $E_{5.8}^{-}$ preparation, obtained by the *in vitro* adenylylation of $E_{2.3}^{-}$ (see Methods), shows a response to L-glutamate that is intermediate between those of $E_{2.3}^{-}$ and E_9^{-}. This suggests that the adenylyltransferase also produces hybrid glutamine synthetase forms under the conditions of the *in vitro* adenylylation reaction. The mixture of unadenylylated and adenylylated enzyme forms for $\bar{n}$ = 9 does not have the same type of L-glutamate saturation curve (Figure 4b). However, the contribution of $E_{1.2}^{-}$ to the mixture causes a distortion of the data obtained with the mixture so that these data are complicated.

Binding of L-*Glutamate to Glutamine Synthetase.* The kinetic studies presented in Figure 4a,b with the Mn^{2+}-activated enzyme forms indicated that unadenylylated Mn-enzyme has a sufficiently high affinity to make equilibrium binding measurements feasible. Accordingly, the equilibrium binding of L-[^{14}C]glutamate to the Mn^{2+}-activated $E_{0.8}^{-}$ preparation was investigated. The results from these studies are presented in Figure 5, together with those from related kinetic studies. The nonsymmetry in the binding curve of Figure 5a suggests that the enzyme binding sites are not saturated with L-glutamate at the highest concentrations of this substrate employed ($\sim 10^{-3}$ M). Unfortunately, the binding methods are not accurate enough to measure L-glutamate binding at higher L-glutamate concentrations. Further, a Scatchard plot of the data could not be used to obtain an extrapolated value for the maximum number of L-glutamate binding sites per enzyme molecule because the binding data of Figure 5a do not follow the mass action law. Instead, there is cooperativity in the binding of L-glutamate to the Mn^{2+}-activated $E_{0.8}^{-}$ preparation. It is of

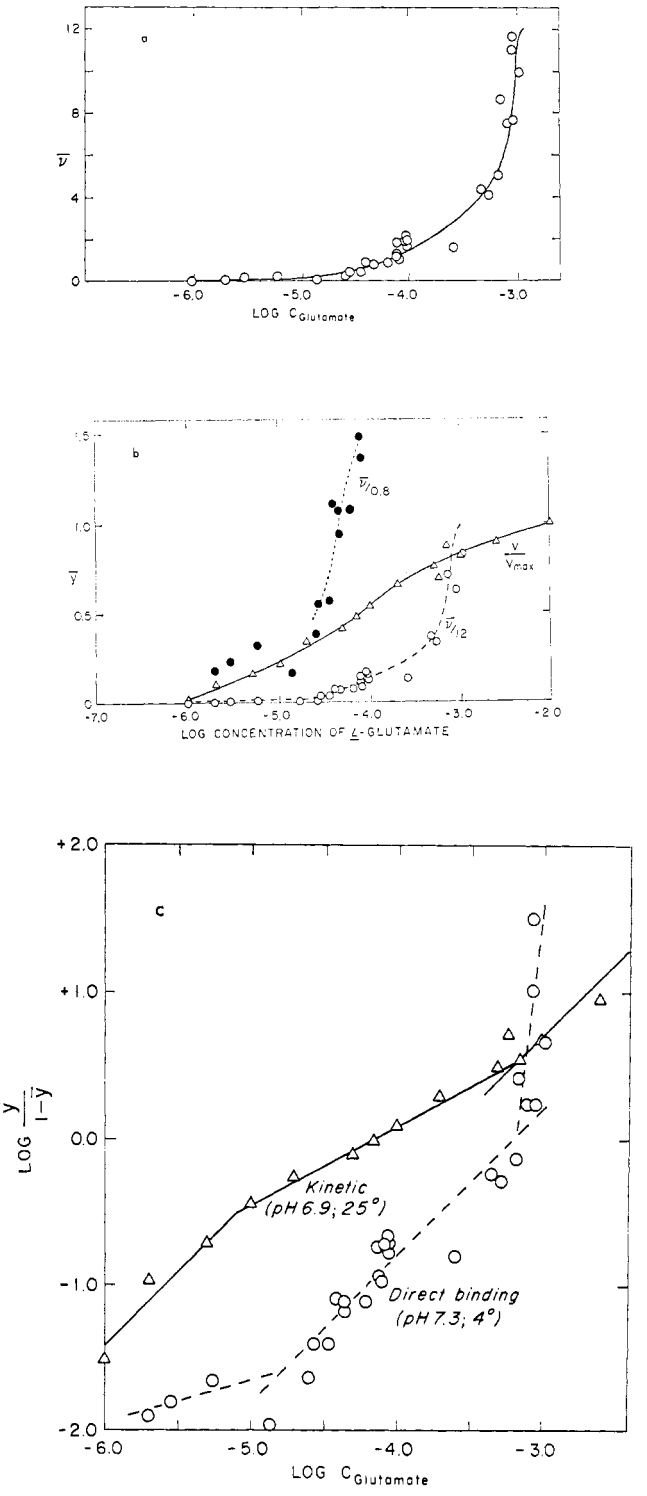

FIGURE 5: The equilibrium binding of L-[^{14}C]glutamate to glutamine synthetase ($E_{0.8}^{-}$) at 4° in 0.02 M imidazole-chloride, 0.10 M KCl, and 0.001 M $MnCl_2$ at pH 7.3 (a) The moles of L-glutamate bound per mole of glutamine synthetase ($\bar{\nu}$) as a function of the logarithm of the free concentration of L-glutamate is plotted. (b) The data of (a) are replotted as saturation functions where $\bar{Y} = \bar{\nu}/12$ (○) or $\bar{\nu}/0.8$ (●), and $\bar{Y} = v/V_{max}$ from kinetic studies at 25° with v and V_{max} determined as in Figure 4. The Mn^{2+}-activated assay system of Table II (Methods) was used with $E_{0.8}^{-}$ and 0.10 M of the imidazole-chloride buffer (pH 6.90) from the binding studies. (c) Hill plots of the data from (b) where $\bar{Y} = \bar{\nu}/12$ from the binding measurements (○) and $\bar{Y} = v/V_{max}$ from the kinetic results (Δ).

interest that $\bar{\nu} \simeq 12$ at a free concentration of $\sim 10^{-3}$ M L-glutamate. Since this number is equal to the number of enzyme subunits (Valentine *et al.*, 1968), it appears that more than one L-glutamate molecule can bind to each subunit. This result differs from that obtained with two of the feedback inhibitors, AMP, and L-tryptophan, in which one molecule of each inhibitor was bound per enzyme subunit (Ginsburg, 1969). Two substrate sites per enzyme subunit possibly explains the inhibition of unadenylylated glutamine synthetase by excess L-glutamate, if one of these L-glutamate sites is an allosteric effector site which affects the reaction of L-glutamate at the catalytic site. The varying kinetic effects of L-glutamate with different Mn–enzyme forms suggest that the binding characteristics of this substrate will be a function of the state of adenylylation of the enzyme. (This was not the case in the binding of the feedback inhibitors, AMP, and L-tryptophan.) However, only the binding of L-glutamate by the $E_{\overline{0.8}}$ preparation was investigated in these studies.

Saturation functions ($\bar{Y}$) of the binding data of Figure 5a are expressed as $\bar{\nu}/0.8$ and $\bar{\nu}/12$ in Figure 5b. Since $E_{\overline{0.8}}$ contains an average of 0.8 adenylyl group per enzyme molecule, $\bar{Y} = \bar{\nu}/0.8$ was plotted to show the saturation of the first 0.8 equiv of L-glutamate. Kinetic data for the saturation ($\bar{Y} = v/V_{max}$) of the Mn^{2+}-activated adenylylated subunits are shown also. The pH difference between the kinetic and binding experiments represents the temperature dependence of the pK' of the imidazole buffer, since the same buffer was used for both studies. Since the ionizing protein groups in the neutral pH range might be expected to be predominantly imidazole groups, these should have a temperature dependence of ionization similar to that of the buffer. Consequently, it was felt to be more correct not to adjust the pH to compensate for the temperature difference between the binding (4°) and kinetic (25°) studies.

At low concentrations of L-glutamate, 0.8 of the L-glutamate binding sites is saturated more quickly with increasing substrate concentrations than the kinetic data show (Figure 5b). As in the case of ATP binding to glutamine synthetase (Denton and Ginsburg, 1970), inactive enzyme–substrate complexes appear to be formed since $\gg 0.8$ equiv of L-glutamate are bound to the Mn^{2+}-activated enzyme ($E_{\overline{0.8}}$).

Hill plots of the kinetic ($\bar{Y} = v/V_{max}$) and binding ($\bar{Y} = \bar{\nu}/12$) data are shown in Figure 5c. If $\bar{Y}$ should equal $\bar{\nu}/24$ for the binding data, the Hill plot is unchanged at <0.5 mM L-glutamate; at higher L-glutamate concentrations, the binding points would not cross zero on the ordinate or half-saturation for $\bar{\nu} < 12$. It is apparent that there is no immediately obvious correlation between the binding and kinetic data, regardless of the way in which the data are plotted. Asymptotes of unity (extrapolated to $K'_m \simeq 0.026$ and 0.2 mM) for the extremes of L-glutamate concentrations are observed in the Hill plots of the kinetic data, with the unit slopes connected by slopes of less than one at intermediate L-glutamate levels. In contrast, the binding data produce a Hill slope of less than one at very low L-glutamate concentrations which becomes unity at higher concentrations of substrate ($\sim$0.02–0.5 mM). The kinetic data for all Mn^{2+}-activated enzyme forms (Figures 4b and 5c) indicate that there is a negative interaction between (Mn) glutamine synthetase and L-glutamate at subsaturating concentrations of this substrate, with Hill plot slopes less than unity in these L-glutamate concentration ranges. However the binding and kinetic data for the interaction of glutamine synthetase with L-glutamate are obviously dissimilar. This could indicate that the substrate ATP, which is absent in the binding measurements, determines the characteristics of the interaction between the Mn–enzyme forms and L-glutamate under assay conditions (see Discussion).

Discussion

To determine if heterologous interactions between adenylylated and unadenylylated subunits influence V_{max}, as well as K'_m for substrates, biosynthetic reaction velocity as a function of the extent of adenylylation ($\bar{n}$) of the enzyme was studied here. It is assumed that the biosynthetic activity of adenylylated subunits in hybrid or homologous molecules are specifically measured by using Mn^{2+} as the activating divalent cation (Figure 2a,b). Conversely, unadenylylated subunits appear to require Mg^{2+} for the expression of biosynthetic activity. The conclusion that adenylylated subunits have no Mn^{2+}-dependent activity is based on the assumption that the divalent cation specificity is maintained whether or not glutamine synthetase is fully adenylylated. The absolute specificity of unadenylylated subunits for Mg^{2+} is concluded, in addition, from an extrapolation of the data for $E_{\overline{0.8}}$–E_{12} to an enzyme form with zero 5′-adenylyl group, rather than on a direct measurement of the Mn^{2+}-dependent activity of E_0. (It may be significant that we have been unable to obtain E_0 for this measurement; perhaps a molecular "core" of one 5′-adenylyl group is of physiological significance.) If it is correct to assume that adenylylated and unadenylylated subunits have absolute requirements for Mn^{2+} and Mg^{2+}, respectively, nonlinear V_{max} and different K'_m values for enzyme forms in the biosynthetic reaction must be attributed to subunit interactions within hybrid enzyme molecules. Alternatively, the specificity of divalent cations could vary slightly as a function of the adenylylation state of the enzyme; subunit interactions would be involved in this case also, although the interpretation of V_{max} and K'_m values would become even more complicated. Without evidence to the contrary, it will be assumed that the divalent cation specificity of each type of subunit is maintained throughout all stages of adenylylation.

In the measurements of the Mg^{2+}-dependent activity of different enzyme preparations, the assay conditions were optimal for all states of adenylylation so that the specific activities of Figure 2a are approximately V_{max} values. Clearly, the optimal expression of Mg^{2+}-dependent biosynthetic activity per unadenylylated subunit is not equivalent in the different enzyme preparations. This result differs from that obtained by Kingdon *et al.* (1967) during the adenylylation of glutamine synthetase in which a linear loss of Mg^{2+}-dependent biosynthetic activity was observed (Stadtman *et al.*, 1968; Holzer *et al.*, 1967). The biosynthetic assay conditions of Woolfolk *et al.* (1966), which measures inorganic phosphate release after a timed incubation at 37°, were used in the studies of Kingdon *et al.* (1967). Since then, it has become apparent that these assay conditions are not optimal for the different enzyme forms. For example, at low states of adenylylation, the enzyme forms have a very high affinity for Mn^{2+} (Denton and Ginsburg, 1969), and $>3 \times 10^{-7}$ M Mn^{2+} introduced with the enzyme into the Mg^{2+}-dependent phosphate assay system markedly inhibits this activity; the expression of Mg^{2+}-dependent activity by unadenylylated subunits in adenylylated enzyme forms is inhibited by high L-glutamate and ATP levels (accompanying paper, Denton and Ginsburg, 1970). If the bio-

synthetic assay conditions of Woolfolk *et al.* (1966) are adjusted appropriately, nearly identical results are obtained from this or the spectrophotometric assay used here. If possible, the latter assay is used since it is preferable to measure initial rates.

In the measurement of Mn^{2+}-dependent biosynthetic activity (Figure 2b), the unadenylylated enzyme forms are inhibited slightly by the L-glutamate concentration required to saturate E_{12} (Figure 4a). This inhibitory effect of L-glutamate is much less apparent at 37° than at 25°. The effect of using excess ATP to Mn^{2+} (7.5:5 mM in the Mn^{2+}-dependent assay of Woolfolk *et al.* (1966)) could not be tested here since the spectrophotometric assay system itself requires divalent cation activation (see Methods). Nevertheless, the 37° curve of Figure 2b, which is approximately representative of V_{max} values, indicates that adenylylated subunits do not express equivalent Mn^{2+}-dependent activities in the different enzyme preparations tested. Again, this result differs from that reported previously (Stadtman *et al.*, 1968).

The results presented in this and in the accompanying paper (Denton and Ginsburg, 1970) strongly suggest that each subunit of the dodecameric glutamine synthetase molecule has catalytic sites for ATP and L-glutamate, and that heterologous interactions between adenylylated and unadenylylated subunits are involved in determining V_{max} and K_m' of each type of subunit. Mn^{2+} or Mg^{2+} specifically activate only adenylylated or unadenylylated subunits, respectively, so that these ions in conjunction with substrates must be capable of determining whether or not inactive or active substrate complexes are formed. The normalized expression of activity for either type of subunit is maximal when the subunit is present as a major or minor component of the enzyme. Also, the hybrid enzyme forms that contain a predominant proportion of either adenylylated or unadenylylated subunits are susceptible to inhibition by L-glutamate. (With $\bar{n} = 9$, unadenylylated subunits are inhibited by high concentrations of L-glutamate in the Mg^{2+}-dependent assay, whereas with $\bar{n} < 2.3$, adenylylated subunits in the Mn^{2+}-dependent assay are inhibited by high levels of L-glutamate.) The binding results suggest the presence of a second L-glutamate binding site per subunit, and this might function as an allosteric effector site.

In studies with the mammalian glutamine synthetase, Meister *et al.* (Levintow and Meister, 1953; Krishnaswamy *et al.*, 1962) have shown that this enzyme reacts first with ATP–Mg and second, with L- or D-glutamate to form an activated glutamyl–enzyme complex. It is certain that the *E. coli* enzyme must first be activated by a specific divalent cation (Kingdon *et al.*, 1968). It is attractive to consider that ATP–metal next reacts with the *E. coli* enzyme and that it is with the ATP–metal–enzyme species that L-glutamate reacts, as in the case of the mammalian enzyme. Indirect evidence supporting a stepwise reaction for the *E. coli* enzyme is suggested by the substrate binding and kinetic results. There is a correlation between the binding and kinetic data with ATP in the affinity of the *E. coli* enzyme for ATP at low concentrations of this substrate in the overall characteristics of the ATP binding. In contrast, the binding results with L-glutamate are quite different from the kinetic saturation functions. Since the binding of L-glutamate to the enzyme is studied in the absence of ATP, it could be that it is the interaction of L-glutamate with the ATP–metal–enzyme complex forms that determines the characteristics of the L-glutamate saturation functions under assay conditions. Although the binding of L-glutamate does not require the presence, or prior binding, of ATP, the Mn–enzyme has a higher affinity for ATP than for L-glutamate when the binding of these substrates are measured separately. The stereospecificity of the interaction of the *E. coli* enzyme with L-glutamate appears to depend upon the enzyme form. Only Mn^{2+}-activated, unadenylylated glutamine synthetase preparations have appreciable activities with the D isomer of glutamate, and these are comparable with that observed with the mammalian enzyme.

Although only the biosynthetic reaction is considered here, it should be mentioned that heterologous subunit interactions are not observed in the γ-glutamyl transfer reaction. Perhaps subunit interactions are uncoupled under the conditions of this reverse reaction so that adenylylated and unadenylylated subunits act as catalytically independent units in the formation of γ-glutamylhydroxamate.

A scheme of substrate binding to the Mn^{2+}-activated glutamine synthetase from *E. coli* is presented in Figure 6. The results from this and the accompanying paper (Denton and Ginsburg, 1970) on the binding of substrates to glutamine synthetase in the forward reaction are combined in the discussion of Figure 6. For simplicity, only one hexagon of the double-hexagon structure (Valentine *et al.*, 1968) is shown. A hybrid molecule is depicted as containing two adenylylated subunits ($*Mn^{2+}$ activated) and four unadenylylated subunits (Mg^{2+} activated). Equilibrium A represents the conversion of the native, taut enzyme into the catalytically inactive, relaxed form by the removal of Mn^{2+} from the protein (see legend to Figure 6). The reverse reaction which involves the addition of specific divalent cations (Mn^{2+} or Mg^{2+}) to the relaxed enzyme has been termed a tightening process, and it is not certain that exactly the same native taut form is produced (Valentine *et al.*, 1968; see Stadtman *et al.*, 1968). Although the binding studies suggested that the tightened form might interact differently with ATP, no significant differences between tightened and taut forms could be detected under assay conditions. The two Mn^{2+} ions per subunit that are bound with the highest affinity are shown since both of these are thought to be involved in catalysis (Denton and Ginsburg, 1969). The binding of the first 12 Mn^{2+} by the enzyme molecule is associated with the rather large conformational change accompanying the activation, burial of sulfhydryl, tyrosyl, and tryptophanyl groups, and decrease in hydrodynamic volume (Shapiro and Stadtman, 1967; Kingdon *et al.*, 1968; Shapiro and Ginsburg, 1968; Denton and Ginsburg, 1969). The second Mn^{2+} bound per subunit might be at an inhibitory site.

Equilibria B and C represent the saturation of a Mn^{2+}-activated, hybrid enzyme form with substrate. The characteristics of the saturation of a relatively unadenylylated glutamine synthetase form with either ATP or L-glutamate indicate a negative interaction in which the substrate is bound with an apparent higher affinity at low than at high concentrations of ATP or L-glutamate. The binding characteristics of ATP to the enzyme may relate to those of Mn^{2+}, whereas the interaction of L-glutamate may involve the ATP–Mn–enzyme complex. In B, the binding at low concentrations of substrate to the high-affinity sites is shown, while C indicates the binding of substrate to the rest of the subunits which are in a lower affinity state. The adenylylated subunits are shown as being randomly distributed between the high-affinity (T_1) and low-affinity (T_2) states so that full activity is not expressed in the Mn^{2+}-activated assay until all adenylylated subunits are sat-

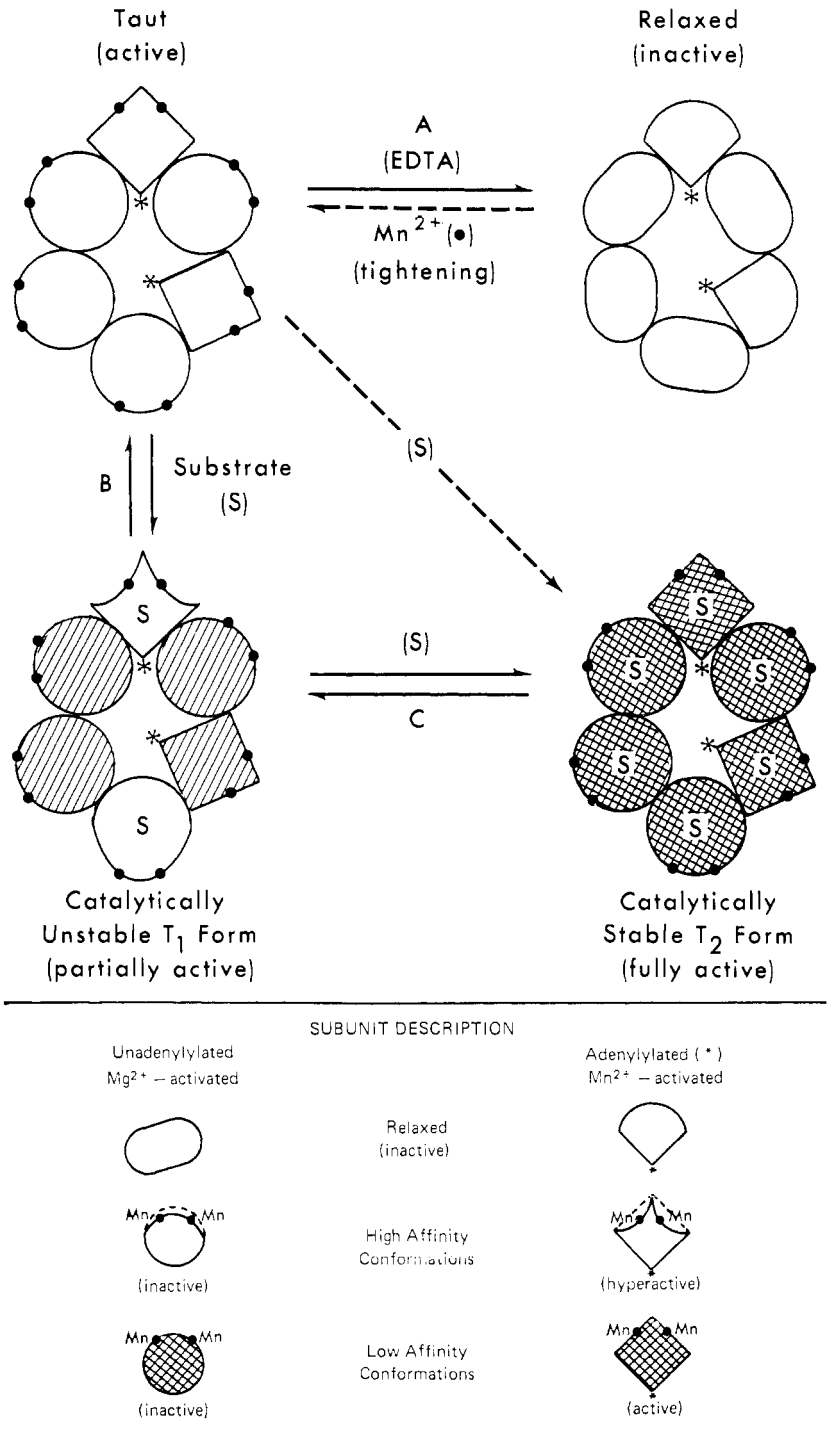

FIGURE 6: Scheme of substrate binding to the Mn^{2+}-activated glutamine synthetase of *E. coli*. The glutamine synthetase molecule is represented as one-half of the actual double-hexagon structure (Valentine *et al.*, 1968) with two adenylylated subunits (*Mn^{2+} activated) and four unadenylylated subunits (Mg^{2+} activated) to form a hybrid molecule. Only one isomer is shown. The enzyme as isolated contains bound Mn^{2+} and is the native taut form; the taut enzyme is converted into a relaxed inactive form by EDTA treatment which may be tightened to an active form by the addition of specific divalent cations (Shapiro and Stadtman, 1967; Kingdon *et al.*, 1968; Shapiro and Ginsburg, 1968). Reaction A, which concerns the binding of Mn^{2+} and the taut to relaxed conversion, has been considered in detail in an earlier paper (Denton and Ginsburg, 1969). Only the two high-affinity Mn^{2+} binding sites per subunit which are thought to be directly involved in catalysis are pictured here. Reactions B and C schematically represent the negative type of interaction observed here in the saturation of the enzyme with substrate, and each might involve multiple transitions. In B, high-affinity sites of adenylylated and unadenylylated subunits are filled with substrate with only a fraction of the total of each type of subunit being in the high-affinity conformation. The product of B is a catalytically unstable T_1 form that converts in reaction C into a stable low-affinity T_2 form, with substrate combining in C with subunits in the low-affinity conformation; included in C is the conformational change of saturated subunits from the high- to low-affinity states so that the T_2 form may contain each type of subunit in the same conformational state. There appear to be differences in the subunit conformations in the T_2 state that are not pictured here which result in non-equivalent V_{max} and L-glutamate K'_m values (but not ATP K'_m values) for either Mn^{2+}-activated or Mg^{2+}-activated subunits (see text). Note that inactive and active subunits bind substrate in both reactions B and C. The dashed arrow indicates that the binding of substrates to the tightened enzyme under some conditions may not go through the same route as does substrate binding to the taut enzyme form. A similar scheme may be used to describe substrate binding to Mg^{2+}-activated enzyme forms, if the activity is considered in this case to reside in only unadenylylated subunits (See text for the discussion of this scheme.)

urated with substrate. Unadenylylated subunits, which are inactive in the Mn^{2+}-dependent assay but appear to stabilize high-affinity adenylylated subunit forms by heterologous subunit interaction, are shown as combining with substrate also. The results from equilibrium binding studies suggest that each subunit binds ATP and L-glutamate. However, in the kinetic analyses, only the saturation of the Mn^{2+}-activated, adenylylated subunits are detected. It seems more consistent from a functional standpoint that the T_1 high-affinity adenylylated subunit forms have higher activities than those of the corresponding T_2 subunit forms. Of course, maximum activity cannot be expressed until all potentially active (adenylylated) subunits are saturated with substrate in the Mn^{2+}-dependent biosynthetic assay.

The product of C in Figure 6 shows the saturated, adenylylated subunits in the same conformation, as defined by these subunits having the same affinity for substrate. This seems to be the case for ATP, but not for L-glutamate. With ATP, all enzyme forms appear to convert into a configuration with the same approximate affinity ($1/K'_m$) for ATP at high concentrations of this substrate, whether or not high-affinity forms for ATP are apparent at low ATP concentrations. The product of B is favored by low adenylylation states in the ATP interaction. In contrast, L-glutamate saturation curves indicate that there are T_1 high-affinity and T_2 low-affinity forms of enzymes in all adenylylation states, with the high- and low-affinity forms both a function of adenylylation. Thus, the affinities of the Mn^{2+}-activated products of each B and C for L-glutamate decrease with increasing extents of adenylylation. Since the fully adenylylated enzyme form has high- and low-affinity sites for L-glutamate, homologous interactions between adenylylated subunits appear to be a factor, in addition to heterologous subunit interactions, in the interaction of L-glutamate with glutamine synthetase.

Subunit interactions also influence the maximum velocity expressed by either type of subunit. Thus, the Mn^{2+}-activated subunits in the T_2 form of Figure 6 are representative of a number of conformations having different affinities for L-glutamate, the same affinity for ATP, and differences in the maximum velocity expressed by each adenylylated subunit. In addition, the different conformations of adenylylated subunits in T_2 forms have a varying capacity to utilize D-glutamate in the biosynthetic reaction. This appears to be related to a unique conformation that adenylylated subunits can assume in a molecule containing few 5'-adenylyl groups. A large number of unadenylylated subunits that are inactive in the Mn^{2+}-dependent assay also makes the adenylylated subunits susceptible to inhibition by L-glutamate (see above). Possibly this is an allosteric effect of additional L-glutamate binding to each subunit which causes a conversion to a T_2 form with a lower V_{max} value.

The interaction of Mg^{2+}-activated subunits with substrate can be considered also in the framework of the scheme of Figure 6, if unadenylylated subunits are considered to be catalytically active and adenylylated subunits to be inactive. Discrete high- and low-affinity forms are not observed in the Mg^{2+}-dependent biosynthetic assay that specifically measures the activity of unadenylylated subunits. However, there are K'_m variations for L-glutamate and ATP with different extents of adenylylation. Higher affinity forms for L-glutamate or ATP appear to be produced by a large proportion of adenylylated subunits which are inactive in the Mg^{2+} assay system. Thus, a predominance of inactive subunits in either the Mg^{2+}- or Mn^{2+}-dependent assay favor active forms that have a higher affinity for ATP and L-glutamate. High states of adenylylation also makes the unadenylylated subunits susceptible to inhibition by L-glutamate (see above).

In studies on the binding of feedback inhibitors to the glutamine synthetase of *E. coli* (Ginsburg, 1969), it was observed that the inhibition of the Mg^{2+}-dependent biosynthetic activity by AMP was independent of the extent of adenylylation of the enzyme preparation. However, the Mn^{2+}-dependent activity was sensitized to AMP inhibition by increasing adenylylation. This effect in the Mn^{2+}-activated biosynthetic assays was most marked at limiting L-glutamate and ammonia with saturating ATP concentrations. Limiting the ATP concentration in either the Mg^{2+} or Mn^{2+} assay systems increased the sensitivity of the enzyme forms toward AMP inhibition, although AMP inhibition is noncompetitive with ATP at low levels of this substrate. If these results are interpreted in terms of the scheme shown in Figure 6, discrete intermediates in the binding of substrates in the Mn^{2+} assay could differ in their sensitivity to AMP inhibition, or AMP might potentiate the T_1 forms. The antagonistic effect of ATP in the AMP inhibition studies suggest that the T_2 form stabilized by ATP has a lower affinity for AMP. Still, the adenylylated Mn–enzyme form that would have the highest fractional activity in the low-affinity T_2 form is most sensitive to AMP inhibition. It could be supposed that the T_2 intermediates in reaction C of Figure 6, that are differentially stabilized by L-glutamate, have different affinities for AMP. Then, the variation in the response of the Mn^{2+}-activated enzyme forms to AMP, as a function of adenylylation, would be expected to be more exaggerated at limiting substrate (ATP or L-glutamate and ammonia) concentrations. Saturating substrate concentrations would favor a more uniform conversion to the corresponding T_2 forms. The lack of resolution of intermediates in the binding of substrates in the Mg^{2+} assay may directly relate to the observation that different Mg^{2+}-activated enzyme forms appear to be equally sensitive toward AMP inhibition. These observations on the inhibition of the biosynthetic activities by AMP do not relate in any obvious way to those on the inhibition of the γ-glutamyl transfer activity of different enzyme forms by AMP (Kingdon *et al.*, 1967; Shapiro *et al.*, 1967).

In this discussion of the negative-type interaction of glutamine synthetase with substrates (ATP and L-glutamate), we have called the T_1 form a high-affinity conformation, and the T_2 form a low-affinity conformation. We shall now apply these same terms to the individual subunits in order to discuss the interaction between T_1 high-affinity and T_2 low-affinity subunits in the same molecule. In the active configurations, the adenylylated subunits (T_1* and T_2*) are combined with substrate in Figure 6 and unadenylylated subunit forms (T_1 and T_2) also are produced by the interaction with substrate. Although the equilibria between these subunits may be thermodynamically described in terms of the same conformations in the absence of substrate, the critical role of substrates in the subunit equilibria should not be forgotten. In a negative type of interaction, an induced change in conformation has to occur. In B of Figure 6, the subunits can exist in equilibria between high- and low-affinity forms before combining with substrate, but substrate combining with the high-affinity form then induces a change in the conformation of neighboring subunits. Alternatively, the substrate itself induces the high-affinity form (Koshland, 1958) which in turn makes the combination of neighboring subunits with substrate more difficult. In either case, the product of B in Figure 6 is the same. In C, the saturation of subunits in a low-affinity configuration with substrate may or may not induce a further conformational change (diagonal to crosshatch in Figure 6), but the saturation of these subunits with substrate must induce a change from high- to low-affinity conformations in the subunits produced by B. With the understanding that substrates are intimately involved in the induced conformational changes, the equilibria between subunit conformations will be written without regard to the saturation with substrates.

The relative proportion of subunits in the T_1 and T_2 states will be determined by subunit interactions. The equilibria be-

tween subunits are a function of the state of adenylylation (see below), but homologous interactions will be considered first. As proposed by Koshland *et al.* (1966) for the type of interaction shown, the three equilibria determining the proportion of subunits in the high-affinity state are $K_{T_1T_1}$, $K_{T_1T_2}$, and $K_{T_2T_2}$. In order to obtain negative interaction as occurs in the binding of substrates to glutamine synthetase, $K_{T_1T_1} < K_{T_2T_2} < K_{T_1T_2}$. It is the negative interaction between high-affinity subunit forms and a positive interaction between subunits having high- and low-affinity forms that allows the enzyme to convert from a high- into a low-affinity state to produce the overall reaction characteristics of a negative-type interaction. Otherwise, all of the subunits would assume the high-affinity conformation as substrate is bound. Since T_1 subunits favor the formation of T_2 neighbors, other T_1 subunits will have a more probable location in a distant portion of the molecule. The electron micrographs of Valentine *et al.* (1968) have shown that the glutamine synthetase molecule is composed of 12 subunits arranged in two superimposed hexagons. Then, each T_1 subunit is in direct contact with at least three other subunits (more, if the hexagons are skewed in relation to each other), and the rest of the subunits may be converted into T_2 subunits by the presence of only a few T_1 subunits. This type of abrupt transition that can occur when a large number of subunits are involved has been described previously by Frieden (1967) for the cooperative model of Monod *et al.* (1965).

The fact that glutamine synthetase may contain two types of subunits, adenylylated (*) and unadenylylated, that undoubtedly differ in conformation adds further complexities to the model of Koshland *et al.* (1966) described above. Each type of subunit can undergo a $T_1 \rightarrow T_2$ transition, and there can be heterologous subunit interaction so that the terms $K_{T_1T_1}$*, $K_{T_1T_2}$*, K_{T_1}*$_{T_2}$, and $K_{T_2T_2}$* plus the above terms for each type of homologous interaction must be taken into account. The binding studies showed that the affinity for ATP at very low ATP concentrations was about that of assayed adenylylated subunits. Then, the affinity of the T_1 and T_1* forms for ATP should be about comparable. Still, the proportion of inactive T_1 subunits (*i.e.*, not Mn^{2+} activated) markedly influences the overall subunit equilibria in the reaction of either ATP or L-glutamate with the Mn^{2+} activated enzyme. Although there is no information on the magnitude of the interaction constants, the general observations suggest that K_{T_1}*$_{T_2}$* > $K_{T_1T_2}$ > $K_{T_1T_2}$* > K_{T_1}*$_{T_2}$.

In summary, subunit interactions in the glutamine synthetase molecule appear to be an important factor in determining the catalytic parameters of this enzyme in the biosynthetic reaction. The dramatic changes produced by the adenylylation of the *E. coli* glutamine synthetase (Holzer *et al.*, 1967; Kingdon *et al.*, 1967), also cause the subtle changes in the response of glutamine synthetase to substrates that have been considered here. Some possible physiological functions for heterologous subunit interactions influencing the affinities of the enzyme forms for substrates were considered in the accompanying paper (Denton and Ginsburg, 1970). The effects of adenylylation on the catalytic Mg^{2+}- and Mn^{2+}-dependent activities are most marked at either extreme of adenylylation. It could be advantageous to the organism to be able to effect the greatest changes in the glutamine synthetase activity by adenylylation or deadenylylation at the extremes of adenylylation states in order to be most responsive to the needs of the cells. At intermediate stages of adenylylation, when the biosynthetic activities of glutamine synthetase are less affected by adenylylation, the cell may not need to anticipate any large changes in metabolic balance.

Acknowledgments

We wish to thank Dr. E. R. Stadtman for many helpful discussions while this work was in progress, and for our collaborative efforts to obtain glutamine synthetase preparations in different adenylylation states.

References

Boch, R. M., Ling, N. S., Morell, S. A., and Lipton, S. H. (1956), *Arch. Biochem. Biophys. 62*, 253.

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.

Denton, M. D., and Ginsburg, A. (1969), *Biochemistry 8*, 1714.

Denton, M. D., and Ginsburg, A. (1970), *Biochemistry 9*, 617.

Dixon, M., and Webb, E. C. (1964), Enzymes, 2nd ed, New York, N. Y., Academic, pp 145–166.

Dreyer, W. J. (1960), *Brookhaven Symp. Biol. 13*, 243.

Frieden, C. (1967), *J. Biol. Chem. 242*, 4045.

Ginsburg, A. (1969), *Biochemistry 8*, 1726.

Ginsburg, A. (1970), *Methods Enzymol. 17* (in press).

Ginsburg, A., and Carroll, W. R. (1965), *Biochemistry 4*, 2159.

Ginsburg, A., and Mehler, A. H. (1966), *Biochemistry 5*, 2623.

Holzer, H., Mecke, D., Wulff, K., Leiss, K., and Heilmeyer, L., Jr. (1967), *Advan. Enzyme Regulation 5*, 211.

Kingdon, H. S., Hubbard, J. S., and Stadtman, E. R. (1968), *Biochemistry 7*, 2136.

Kingdon, H. S., Shapiro, B. M., and Stadtman, E. R. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 1703.

Kingdon, H. S., and Stadtman, E. R. (1967), *Biochem. Biophys. Res. Commun. 27*, 470.

Koshland, D. E., Jr. (1958), *Proc. Natl. Acad. Sci. U. S. 44*, 98.

Koshland, D. E., Jr., Nemethy, G., and Filmer, D. (1966), *Biochemistry 5*, 365.

Krishnaswamy, P. R., Pamiljans, V., and Meister, A. (1962), *J. Biol. Chem. 237*, 2932.

Layne, E. (1957), *Methods Enzymol. 3*, 447.

Levintow, L., and Meister, A. (1953), *J. Am. Chem. Soc. 75*, 3039.

Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12*, 88.

Schauenstein, E., and Bayzer, H. (1955), *J. Polymer Sci. 16*, 45.

Scatchard, G. (1949), *Ann. N. Y. Acad. Sci. 51*, 660.

Shapiro, B. M., and Ginsburg, A. (1968), *Biochemistry 7*, 2153.

Shapiro, B. M., Kingdon, H. S., and Stadtman, E. R. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 642.

Shapiro, B. M., and Stadtman, E. R. (1967), *J. Biol. Chem. 242*, 5069.

Shapiro, B. M., and Stadtman, E. R. (1968a), *Biochem. Biophys. Res. Commun. 30*, 32.

Shapiro, B. M., and Stadtman, E. R. (1968b), *J. Biol. Chem. 243*, 3769.

Shapiro, B. M., and Stadtman, E. R. (1970), *Methods Enzymol. 17* (in press).

Stadtman, E. R., Shapiro, B. M., Ginsburg, A., Kingdon, H. S., and Denton, M. D. (1968), *Brookhaven Symp. Biol. 21*, 378.

Valentine, R. C., Shapiro, B. M., and Stadtman, E. R. (1968), *Biochemistry 7*, 2143.
Woolfolk, C. A., Shapiro, B. M., and Stadtman, E. R. (1966), *Arch. Biochem. Biophys. 116*, 177.
Wulff, K., Mecke, D., and Holzer, H. (1967), *Biochem. Biophys. Res. Commun. 28*, 740.
Wyman, J. (1964), *Advan. Protein Chem. 19*, 223.
Wyman, J. (1967), *J. Am. Chem. Soc. 89*, 2202.

Mechanism of the Oxidative Dephosphorylation of the Phosphoprotein Phosvitin*

Robert W. Rosenstein† and George Taborsky

ABSTRACT: The mechanism of the oxidative dephosphorylation of the egg yolk phosphoprotein phosvitin (Grant, C. T., and Taborsky, G. (1966), *Biochemistry 5*, 544) has been elucidated on the basis of isotopic tracer experiments. Essentially all of the more than 100 phosphoserine residues of phosvitin appear to be potentially oxidizable in a random and independent manner. The stable products of the oxidation are aminomalonic semialdehyde residues and inorganic orthophosphate. Although the overall reaction must pass through a transient state corresponding to the oxidation level of the enol phosphate derivative of phosphoserine, a direct search failed to yield evidence for the production of a stable phosphoenol (which was postulated in the earlier work cited above) and the demonstration of the stability of the C–H bond at the α-carbon of the oxidized residues ruled it out. The results, including the finding that phosphate release occurs by P–O bond cleavage, are wholly consistent with a mechanism by which an oxidatively generated carbonium ion derivative of phosphoserine is converted into a stable product by the direct formation of the free aldehyde and a monomeric metaphosphate ion, the latter reacting with water to yield inorganic orthophosphate. The possible biological significance of the oxidative activation of phosphoprotein ester groups is seen in the hypothetical action of phosvitin as an energy source during embryonic development in the hen egg, or in its service as a macromolecular model for the formation of a chemical intermediate in the energy conservation step of oxidative phosphorylation.

When various phosphate esters, including phosphoproteins, interact with Fe^{2+} and O_2, the metal and the phosphorylated ligand become oxidized: results obtained with phosvitin suggested that the oxidation of the protein occurs by α,β dehydrogenation of its phosphoser ineresidues, yielding the corresponding enolphosphate, followed by hydrolytic formation of P_i and the aldehyde derivative of serine, aminomalonic semialdehyde (Grant and Taborsky, 1966). Prompted by our interest in the possible biological significance of this reaction (see Discussion), we undertook an investigation of its chemical mechanism.

Experimental Procedure

Materials. Phosvitin was prepared according to Joubert and Cook (1958). It was analyzed for N, P, and Fe as before (Connelly and Taborsky, 1961), with results very similar to those of Mecham and Olcott (1949). The protein was rendered metal free and stored as described earlier (Taborsky, 1963).

[^{3}H]Water (New England Nuclear; 25 Ci/l.) was diluted for use up to twofold. [^{18}O]Water (Bio-Rad; 10 atom % excess) was diluted as given in Results. [^{32}P]ATP was prepared with carrier-free [^{32}P]P_i (Tracerlab) by the method of Glynn and Chappell (1964). At the time of its use, its specific activity was about 2×10^7 cpm/μmole. Several batches of [^{3}H]sodium borohydride (Nuclear-Chicago) had nominal specific activities of 80–500 Ci/mole. It has been the experience in our laboratory (T. S. Stashwick, 1967, personal communication) that [^{3}H]$NaBH_4$ may contain an appreciable fraction of acid-stable counts. The acid stability may be noted with the dry material as received, or it may develop upon cold storage in strongly alkaline solution. We store the reagent in the dry form and use only such preparations which have an acid-stable isotope content of no more than a few per cent. Its specific activity, in terms of its active H content, was measured on the basis of the ^{3}H content of lactic acid prepared from it and pyruvic acid. (The [^{3}H]lactic acid was purified according to Busch *et al.* (1952) and was assayed by the enzymic procedure of Lundholm *et al.* (1963).) The specific activity of [^{3}H]$NaBH_4$, when used, always exceeded 50 Ci/mole; any dilutions were made with $NaBH_4$ (Alfa Inorganics).

Gu·HCl (Ultra Pure; Mann), DPN (P-L Biochemicals), and lactic dehydrogenase (Sigma) were commercial products.

* From the Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06520. *Received September 9, 1969.* Most of the data in this paper are taken from the dissertation presented by Robert W. Rosenstein to the faculty of the Graduate School of Yale University in partial fulfillment of the requirements for the degree of Doctor of Philosophy. The work was done in the former Department of Biochemistry of Yale University and was supported by a research grant from the U. S. Public Health Service (AM 03188).

† Predoctoral Trainee of the U. S. Public Health Service, 1964–1969. Present address: Department of Microbiology, College of Medicine, University of Illinois, Chicago, Ill. 60680.