Escherichia coli Glutamine Synthetase. Determination of Rate-Limiting Steps by Rapid-Quench and Isotope Partitioning Experiments†

Thomas D. Meek, Kenneth A. Johnson, and Joseph J. Villafranca*,‡

Appendix: Derivation of Equations for Isotope Partitioning Experiments for a Terreactant Enzyme

T. D. Meek and J. J. Villafranca

ABSTRACT: The ATPase and biosynthetic reactions of unadenylylated *Escherichia coli* glutamine synthetase have been studied by using rapid-quench, stopped-flow, and isotope partitioning techniques. The time course of the ATPase reaction (24 °C) is characterized by a "burst" of acid-labile phosphate equivalent to 0.67 mol of enzyme, which is followed by a slower steady-state phase. In the presence of Mg^{2+}, only unadenylylated subunits are capable of producing the observed burst. The rate constant for the transient phase is 10.3 s^{-1}, which is considerably faster than the turnover number of the ATPase reaction (0.011 s^{-1}). A similar burst, equivalent to 0.57 mol of enzyme, is observed in the time course of the biosynthetic reaction at 10 °C, in which the transient rate constant of 88 s^{-1} is also much faster than the turnover number of the biosynthetic reaction (4.0 s^{-1}, 10 °C). When these data are combined with the results of positional isotope exchange of [γ-^{18}O]ATP [Midelfort, C. F., & Rose, I. A. (1976) *J. Biol. Chem. 251*, 5881–5887], it is shown that the enzyme-bound intermediate responsible for the burst of acid-labile phosphate is γ-glutamyl phosphate. In the biosynthetic reaction pathway, γ-glutamyl phosphate is formed *after* NH_4^+ binds to the enzyme, and the presence of NH_4^+ in this quaternary E–MgATP–Glu–NH_4^+ complex effects an increase in the rate of phosphoryl group transfer that is substantially faster than that of the E–MgATP–Glu complex in the ATPase reaction. Because it is formed and consumed more rapidly than the net rate of catalysis, γ-glutamyl phosphate is a kinetically competent intermediate of both reaction pathways. At 10 °C, an identical transient phase (rate constant = 96 s^{-1}) is observed by measuring changes in protein fluorescence intensity with the stopped-flow technique, which indicates that the chemical formation of γ-glutamyl phosphate is accompanied by a change in enzyme conformation. From isotope partitioning experiments, it is found that MgATP is "sticky" in the binary E–MgATP complex ($k_{off} = 0.2V_1/E_t = 2.9$ s^{-1}) and is infinitely sticky in the E–MgATP–Glu and E–MgATP–Glu–NH_4^+ complexes. Since the rate of MgATP release from E–MgATP is nearly twice the turnover number of the reverse biosynthetic reaction ($V_2/E_t = 1.65$ s^{-1}), MgATP release is not solely rate limiting for the reverse reaction. Glutamate dissociates from E–MgATP–Glu at a rate of 19 s^{-1} ($1.3V_1/E_t$) and is infinitely sticky in the E–MgATP–Glu–NH_4^+ complex. Glutamate dissociates so rapidly from the E–Glu complex as to render it insignificant to the biosynthetic reaction pathway. These results are in accord with a kinetic mechanism that is predominantly ordered [Meek, T. D., & Villafranca, J. J. (1980) *Biochemistry 19*, 5513–5519]. From the results of isotope partitioning in the reverse reaction, it is shown that the rate-limiting step of the forward biosynthetic reaction is the release of MgADP from E–MgADP. The rate constant for this dissociation is 14 s^{-1}, which equals V_1/E_t and is 8 times the value of V_2/E_t.

Glutamine synthetase from *Escherichia coli* consists of 12 identical subunits and has a molecular weight of 600 000. It exists in both unmodified and covalently modified forms in which the modification is enzymatic adenylylation of a single tyrosyl residue per subunit (Stadtman & Ginsburg, 1974). In addition to the biosynthesis of glutamine (eq 1), glutamine

$$\text{MgATP} + \text{L-Glu} + NH_4^+ \rightarrow \text{MgADP} + \text{L-Gln} + P_i \quad (1)$$

synthetase also catalyzes a glutamate-dependent ATPase reaction whose products are MgADP, P_i, and the lactam derivative of glutamic acid, pyrrolidonecarboxylate (eq 2)

$$\text{MgATP} + \text{L-Glu} \rightarrow \text{MgADP} + P_i + \text{pyrrolidonecarboxylate} \quad (2)$$

(Weisbrod & Meister, 1973). Both reactions require two divalent cations per subunit, and in the presence of Mg^{2+}, only unadenylylated subunits are active (Stadtman & Ginsburg, 1974).

It has been proposed that the mechanism of either reaction involves the initial formation of a γ-glutamyl phosphate intermediate from MgATP and glutamate (eq 3), from which

$$\text{MgATP} + \text{Glu} \rightarrow \text{MgADP}\cdot\gamma\text{-Glu-P} \quad (3)$$

the activated phosphate group is displaced by ammonia in the biosynthetic reaction (eq 4) and by the α-amino group of

$$\text{MgADP}\cdot\gamma\text{-Glu-P} + NH_3 \rightarrow \text{MgADP} + \text{Gln} + P_i \quad (4)$$

glutamate in the ATPase reaction (eq 5) (Meister, 1974).

$$\text{MgADP}\cdot\gamma\text{-Glu-P} \rightarrow \text{pyrrolidonecarboxylate} + P_i + \text{MgADP} \quad (5)$$

Although there is a plethora of convincing evidence from chemical trapping studies that enzyme-bound γ-glutamyl phosphate is formed in the absence of ammonia (Krishnaswamy et al., 1962; Tsuda et al., 1971; Todhunter & Purich, 1975; Gass & Meister, 1970), this intermediate cannot be detected in the full biosynthetic reaction by using similar methods, presumably due to the swift intervention of ammonia. On the basis of isotope exchange studies, Wedler & Boyer (1972) have proposed that the mechanism of the biosynthetic reaction is concerted, therefore involving no acyl phosphate intermediate. However, more recently Midelfort & Rose (1976) have concluded from studies using the positional isotope

† From the Departments of Chemistry (T.D.M. and J.J.V.) and Biochemistry (K.A.J.), The Pennsylvania State University, University Park, Pennsylvania 16802. *Received September 22, 1981.* Supported in part by the U.S. Public Health Service (GM23529 to J.J.V. and GM26726 to K.A.J.). A preliminary report of these data has been previously presented (Meek et al., 1981).

‡ J.J.V. is an Established Investigator of the American Heart Association.

0006-2960/82/0421-2158$01.25/0

exchange technique that γ-glutamyl phosphate is indeed an intermediate of both reaction pathways.

In addition to chemical proof, it is necessary to demonstrate that a putative intermediate is kinetically competent: that its rates of formation and decomposition on the reaction pathway are greater than or equal to the rate-limiting step for overall enzymatic catalysis. This is usually accomplished with a pre-steady-state kinetic method in which the transient existence of the enzyme-bound intermediate can be directly observed and measured by using large amounts of enzyme.

In this report rapid-quench techniques and stopped-flow fluorescence measurements are used to elucidate the chemical events that occur in the pre-steady-state phase of both the ATPase and biosynthetic reactions. From the rate constants derived from these experiments and the findings of Midelfort and Rose, we are able to conclude that γ-glutamyl phosphate is a kinetically competent intermediate of both reaction pathways. Also, isotope partitioning experiments (Rose et al., 1974) are conducted to determine the relative rates of desorption of several of the substrates from their respective enzyme–substrate transitory complexes. The results of these studies allow us to identify the rate-limiting step of the biosynthetic reaction. All of the present data are in concert with a kinetic mechanism in which the substrates and products bind to and are released from the enzyme by a preferred order, as has been determined from a steady-state kinetic treatment of glutamine synthetase (Meek & Villafranca, 1980).

Experimental Procedures

Materials and Methods. Glutamine synthetase was isolated in pure form from *Escherichia coli* in several states of adenylylation (average values of either 1.1, 1.8, or 8.0 adenyl groups per 12 subunits) by the procedure of Shapiro & Stadtman (1970). The enzyme activity and state of adenylylation (spectrophotometric assay) were determined by the procedures of Shapiro & Stadtman (1970). Protein concentrations were measured by the method of Lowry et al. (1951) and by spectral measurements as described by Ginsburg et al. (1970). For studies of the ATPase reaction, endogenous ammonia was removed from enzyme preparations as thoroughly as possible by the method of Pamiljans et al. (1962). [γ-^{32}P]ATP (45 Ci/mmol), L-[U-^{14}C]glutamic acid (293 mCi/mmol), and [8-^{14}C]ADP (50 mCi/mmol) were obtained from New England Nuclear. For the rapid-quench study performed at 10 °C, [γ-^{32}P]ATP was freshly prepared by the method of Schendel & Wells (1973). All other biochemicals were Sigma products.

Determination of Endogenous Ammonia in ATPase Reaction Mixtures. All solutions of substrates and buffers used in rapid-quench studies of the ATPase reaction were made with highly purified water obtained from a Millipore Super-Q ultrapure water system. Determination of NH_4^+ by glutamate dehydrogenase (Levitzki, 1970) revealed that endogenous ammonia concentrations were less than 10 nmol/mL in each solution. For detection of even smaller amounts of endogenous ammonia, a more sensitive assay was used. [^{14}C]Glutamate and [γ-^{32}P]ATP were added to two separate mixtures of substrates for the ATPase reaction both before and after endogenous ammonia had been consumed by preincubation of the mixtures with micromolar levels of glutamine synthetase. The [^{14}C]glutamine and $^{32}P_i$ formed were isolated and analyzed as described below. The concentration of endogenous ammonia was about 2 μM (0.8 nmol/mL of reaction mixture), which is equal to about 20% of the enzyme concentration used in typical reaction mixtures. Experimental results are corrected for this amount.

Enzyme Assays. Initial velocities of the ATPase and biosynthetic reactions of glutamine synthetase were measured spectrophotometrically as previously described (Meek & Villafranca, 1980). At 10 °C, the initial rates were measured by the colorimetric determination of phosphate (Woolfolk et al., 1966). Turnover numbers for all reactions were calculated on the basis of a molecular weight of 50000 g/mol of unadenylylated subunit.[1]

Rapid-Quench Experiments. Rapid-quench studies of the ATPase reaction were conducted at 23–24 °C. A 0.2-mL solution containing 50 mM Hepes[2] (pH 7.5), 0.1 M KCl, 10 mM $MgCl_2$, and either 27.1 μM glutamine synthetase ($E_{(1.8)}$;[3] 23.1 μM unadenylylated subunits) or 40.4 μM glutamine synthetase ($E_{(8.0)}$; 13.5 μM unadenylylated subunits) was quickly added by a Gilson automatic pipet to a rapidly mixing 0.2-mL solution containing 50 mM Hepes (pH 7.5), 0.1 M KCl, 10 mM $MgCl_2$, 1.0 mM [γ-^{32}P]ATP (4690 cpm/nmol), and 40 mM glutamate. At various time intervals the reaction was quenched by the addition of 0.4 mL of 0.5 N HCl, and a 0.4-mL aliquot of the quenched reaction mixture was added to 1.0 mL of 0.1 N HCl containing 30 mg/mL acid-washed Norit to absorb unreacted nucleotide. This mixture was then vigorously mixed and centrifuged, and a 0.14-mL aliquot of the supernatant solution was counted for ^{32}P activity. A blank contained enzyme which had been denatured with acid prior to its combination with the substrates.

For analysis of the ATPase reaction at reaction times faster than 3 s, a modified Durrum Multi-Mixer apparatus was used (Benkovic et al., 1974). A solution containing 22.8 μM glutamine synthetase, 50 mM Hepes (pH 7.5), 10 mM $MgCl_2$, and 0.1 M KCl was added with rapid mixing to an equal volume of a solution consisting of 1.0 mM [γ-^{32}P]ATP (920 cpm/nmol), 40 mM glutamate, 50 mM Hepes (pH 7.5), 10 mM $MgCl_2$, and 0.1 M KCl. At various times the reaction was quenched with 2 volumes of 0.5 N HCl. A 0.3-mL aliquot of the quenched solution was added to 2.0 mL of 0.1 N HCl containing 30 mg/mL charcoal. After vigorous mixing and centrifugation, an aliquot of the supernatant solution was counted for ^{32}P activity. A blank contained denatured enzyme.

Similarly, the biosynthetic reaction was studied at 24 °C by using the Durrum Multi-Mixer apparatus. A solution containing 22.8 μM glutamine synthetase, 50 mM Hepes (pH 7.5), 10 mM $MgCl_2$, and 0.1 M KCl was rapidly combined with an equal volume of a solution containing 10 mM [γ-^{32}P]ATP (650 cpm/nmol), 40 mM glutamate, 20 mM NH_4Cl, 10 mM $MgCl_2$, 50 mM Hepes (pH 7.5), 0.1 M KCl, 0.05 mg/mL pyruvate kinase, and 2 mM PEP.[4] At various times the reaction was quenched with 2 volumes of 0.5 N HCl. ^{32}P activity was analyzed as described above. A blank contained denatured enzyme.

At 10 °C, the time course of the biosynthetic reaction was followed at reaction times less than 100 ms by analysis of both phosphate formation and changes in protein fluorescence intensities. For both methods, temperature was maintained at 10.0 ± 0.5 °C with a Lauda circulating constant temperature bath. The rapid-quench experiment was performed on a rapid mixing apparatus (K. A. Johnson, unpublished results) con-

[1] Unless indicated otherwise, all enzyme molarities presented in this paper refer to the concentration of unadenylylated subunits.

[2] Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PEP, phosphoenolpyruvate; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid.

[3] The subscript refers to the average number of adenylylated subunits per dodecamer.

[4] Pyruvate kinase and PEP are present to prevent the accumulation of ADP, a potent product inhibitor.

structed in this laboratory with a design similar to one described by Lymn & Taylor (1970). A solution consisting of 24.3 μM glutamine synthetase, 15 mM $MgCl_2$, 50 mM Hepes (pH 7.5), and 0.1 M KCl was mixed with an equal volume of solution containing 10 mM [γ-^{32}P]ATP (0.136 μCi/μmol), 40 mM glutamate, 20 mM NH_4Cl, 15 mM $MgCl_2$, 50 mM Hepes (pH 7.5), and 0.1 M KCl. The reaction was quenched after various times with 2 volumes of 2 N perchloric acid, and the resulting 0.8-mL mixture was added to a 0.5-mL solution of 60 mM phosphoric acid–10 mM sodium pyrophosphate. Product $^{32}P_i$ was isolated according to the method of K. A. Johnson (unpublished results). A 1.0-mL aliquot of the quenched reaction mixture was placed on a 2.0-mL column consisting of activated charcoal (50–200 mesh, stored under 60 mM H_3PO_4) topped with a 0.2-mL layer of acid-washed Norit. $^{32}P_i$ was eluted with 15 mL of 60 mM H_3PO_4–10 mM $Na_4P_2O_7$ and counted by the Cerenkov method. An additional 0.2-mL aliquot of the quenched reaction mixture was added to 15 mL of 60 mM H_3PO_4–10 mM $Na_4P_2O_7$ and counted as a standard. A blank contained denatured enzyme.

Fluorescence measurements were made by using a stopped-flow apparatus constructed in this laboratory (K. A. Johnson, unpublished results). The apparatus was equipped with a 295-nm excitation source for tryptophan fluorescence. The emission spectrum was observed perpendicular to the excitation beam at 334 nm, and the data were collected and processed by using a 3820 Data Acquisition System from On-Line Instrument Systems. A 0.1-mL solution containing 24.3 μM glutamine synthetase, 15 mM $MgCl_2$, 50 mM Hepes (pH 7.5), and 0.1 M KCl was rapidly combined in a cuvette (2-mm path length) with a 0.1-mL solution containing 10 mM ATP, 40 mM glutamate, 20 mM NH_4Cl, 15 mM $MgCl_2$, 50 mM Hepes (pH 7.5), and 0.1 M KCl, and fluorescence data were collected for 60 ms. A blank containing no substrates was used to generate a base line for the fluorescence intensity.

Isotope Partitioning Experiments. Isotope partitioning studies were conducted at 19–22 °C according to the method of Rose et al. (1974). For ATP, a 0.1-mL solution consisting of 5.3 nmol of glutamine synthetase, 0.5 mM [γ-^{32}P]ATP (23 300 cpm/nmol), 12 mM $MgCl_2$, 50 mM Hepes (pH 7.5), and 0.1 M KCl was rapidly added by pipet to a vigorously stirred 2.0-mL solution of 12.5 mM unlabeled ATP, 24.5 mM $MgCl_2$, 1 mM NH_4Cl, 50 mM Hepes (pH 7.5), 0.1 M KCl, and varying concentrations of glutamate. The reaction was terminated after 3 s with 0.2 mL of 8.5 N perchloric acid, and $^{32}P_i$ was isolated and analyzed as described above. A blank was run for each glutamate concentration in which an identical amount of [γ-^{32}P]ATP was present in the 2.0-mL solution and absent in the solution which contained enzyme.

Isotope trapping of glutamate in both the binary E–Glu and the ternary E–MgATP–Glu complexes was investigated. In the former case, a 0.1-mL solution containing 11.2 nmol of glutamine synthetase, 5 mM [^{14}C]glutamate (8500 cpm/nmol), 50 mM Hepes (pH 7.5), 0.1 M KCl, and 10 mM $MgCl_2$ was rapidly added by pipet to a 5.0-mL solution containing 100 mM unlabeled glutamate, 2 mM ATP, 9.8 mM NH_4Cl, 10 mM $MgCl_2$, 50 mM Hepes (pH 7.5), and 0.1 M KCl. The reaction was stopped after 3 s by the rapid addition of 1.0 mL of 0.1 M EDTA. A blank was run in which all of the [^{14}C]glutamate was present in the 5.0-mL solution.

Analysis of isotope trapping in the ternary E–MgATP–Glu complex required a more sophisticated method. Once formed, E–MgATP–Glu* should rapidly equilibrate with E–MgADP–Glu*~P, a complex in which all of the Glu*~P would be trapped as Gln* by NH_4^+. It was necessary then to bring freshly formed E–MgATP–Glu* into contact with unlabeled glutamate and NH_4^+ before it could isomerize. This was accomplished by using the homemade rapid mixing apparatus. At 25 °C, a solution containing 22.0 μM glutamine synthetase, 50 mM Hepes (pH 7.5), 0.1 M KCl, and 10 mM $MgCl_2$ was rapidly mixed with an equal volume of a solution of 10 mM ATP, 10 mM [^{14}C]glutamate (520 cpm/nmol), 10 mM $MgCl_2$, 50 mM Hepes (pH 7.5), and 0.1 M KCl. After 8.5 ms, the resulting solution was mixed with an equal volume of a solution containing 200 mM unlabeled glutamate, 5 mM ATP, 10 mM $MgCl_2$, 50 mM Hepes (pH 7.5), 0.1 M KCl, and varying levels of NH_4Cl. The combined mixture was collected, and the reaction was terminated after 5 s with 0.2 mL of 0.1 M EDTA. A blank was run for each NH_4^+ concentration in which the [^{14}C]glutamate was present in the solution that contained NH_4^+. [^{14}C]Glutamine was separated from unreacted [^{14}C]glutamate by the method of Prusiner & Milner (1970). A 0.6-mL aliquot of quenched reaction mixture was placed on a 0.5 × 6.0 cm column consisting of Dowex 1-Cl (100–200 mesh) which had been equilibrated with 10 mM imidazole (pH 7.0). [^{14}C]Glutamine was eluted with 3 mL of 30 mM carrier glutamine–10 mM imidazole (pH 7.0). Recovery of glutamine was 90%.

Isotope partitioning of the reverse biosynthetic reaction was studied by using [^{14}C]ADP. A 0.1-mL solution of 5.8 nmol of glutamine synthetase, 2 mM [^{14}C]ADP (8650 cpm/nmol), 10 mM $MgCl_2$, 50 mM Hepes (pH 7.5), and 0.1 M KCl was rapidly added to a vigorously stirred 2.0-mL solution containing 25 mM unlabeled ADP, 100 mM phosphate, 30 mM $MgCl_2$, 50 mM Hepes (pH 7.5), 0.1 M KCl, and varying levels of glutamine. After 10 s, the reaction was quenched with 1.0 mL of 0.1 M EDTA. [^{14}C]ATP was separated from unreacted [^{14}C]ADP by the method of Wedler (1974). A 0.3-mL aliquot of the quenched reaction mixture was placed on a 0.5 × 7.0 cm column consisting of DEAE-cellulose (formate) (Whatman DE52). ADP was eluted first with 6 mL of 0.05 N HCl, followed by elution of ATP with 4 mL of 0.5 N HCl (>95% of the [^{14}C]ATP was recovered). A blank was run for each glutamine concentration in which the [^{14}C]ADP was present in the 2.0-mL solution.

Data Analysis. Steady-state kinetic data were analyzed by using the Fortran programs of Cleland (1967). Initial velocity data yielding intersecting patterns were fitted to eq 6.

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \quad (6)$$

Maximum velocities and apparent Michaelis and dissociation constants of the biosynthetic reaction were taken from computer fits of previously published data (Meek & Villafranca, 1980). Experimental data from isotope partitioning studies were fitted to eq 7 (Raushel & Cleland, 1977).

$$v = \frac{VA}{K + A} \quad (7)$$

The nomenclature used in this paper is that of Cleland (1963).

Transient rate constants for single exponential "bursts" were evaluated similar to the method of Shafer et al. (1972) by fitting experimental data to eq 8, in which P_t is the micromolar

$$P_t = k_{ss}E_t(t) + \beta(1 - e^{-\lambda t}) \quad (8)$$

concentration of radioactive inorganic phosphate produced at time t, k_{ss} is the steady-state rate constant of the reaction at the substrate concentrations given, E_t is the micromolar concentration of unadenylylated subunits, β is the transient burst amplitude in micromolar concentration, and λ is the transient rate constant. For transient phases observed by fluorescence

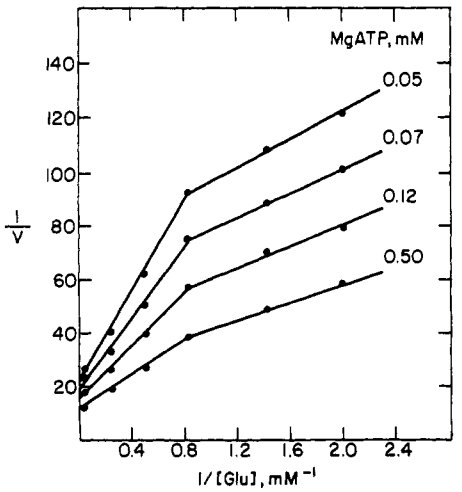


FIGURE 1: Initial velocity pattern of the ATPase reaction at pH 7.5, 25 °C. Glutamate concentrations are varied from 0.5 to 20.0 mM with changing fixed MgATP concentrations of 0.05, 0.07, 0.12, and 0.5 mM. The enzyme concentration is 6.3 μM. Other experimental conditions are the same as given for the standard spectrophotometric assay. The velocities are in arbitrary units.

measurements, rate constants were obtained by fitting the exponential curves according to the method of Dyson & Isenberg (1971).

Results

Steady-State Kinetics. The initial velocity pattern of MgATP vs. Glu for the ATPase reaction is shown in Figure 1. A biphasic pattern is obtained which consists of two linear branches (0.5–1.2 mM glutamate and 1.2–20 mM glutamate). When each of the linear portions is fitted to eq 6, V/E_t = (5.6 ± 0.2) × 10^{-3} s^{-1}, K_{MgATP} = 0.128 ± 0.009 mM, and K_{Glu} = 0.77 ± 0.05 mM at 0.5–1.2 mM glutamate, and V/E_t = (1.14 ± 0.03) × 10^{-2} s^{-1}, K_{MgATP} = 79 ± 5 μM, $K_{i(MgATP)}$ = 70 ± 10 μM, K_{Glu} = 3.1 ± 0.2 mM, and $K_{i(Glu)}$ = 2.7 ± 0.4 mM at 1.2–20 mM glutamate. Due to the biphasic nature of the double-reciprocal plot, these Michaelis and dissociation constants represent limiting values for the "true" constants. However, $K_{i(MgATP)}$ and $K_{i(Glu)}$ from the region of higher glutamate concentrations agree with dissociation constants determined from fluorometric titrations (Timmons et al., 1974). The kinetic constants at high glutamate concentrations are used in all calculations since all studies of the ATPase reaction were conducted at 20 mM glutamate. For the biosynthetic reaction, V_1/E_t = 14.5 ± 0.1 s^{-1} and 4.0 ± 0.4 s^{-1} at 25 and 10 °C, respectively, and V_2/E_t = 1.65 ± 0.28 s^{-1} at 25 °C. From previous kinetic data, $K_{NH_4^+}$ = 0.22 ± 0.01 mM, K_{Glu} = 4.5 ± 0.3 mM, $K_{i(Glu)}$ = 4 ± 1 mM, $K_{i(MgATP)}$ = 0.24 ± 0.06 mM,[5] $K_{i(MgADP)}$ = 0.12 ± 0.04 mM, and K_{Gln} = 2.5 ± 0.2 mM for the biosynthetic reaction at 25 °C (Meek & Villafranca, 1980).

Rapid-Quench Experiments. (*A*) *ATPase Reaction.* The results of rapid-quench studies of the ATPase reaction are shown in Figures 2 and 3. In Figure 2, the time courses of both of the manually quenched reactions are characterized by a burst of acid-labile phosphate which equals 6.8 and 3.4 μM P_i for $E_{\langle 1.8\rangle}$ and $E_{\langle 8.0\rangle}$, respectively. These burst amplitudes are not increased at higher MgATP concentrations. The ratio of burst height to unadenylylated subunit concentration is

[5] The discrepancy between the values of $K_{i(MgATP)}$ for the biosynthetic and ATPase reaction is thought to arise from the nonspecific interaction of NH_4^+ with the enzyme. NH_4^+ is known to exert cooperative binding effects on the *E. coli* enzyme (Bild & Boyer, 1980; Meek & Villafranca, 1980).

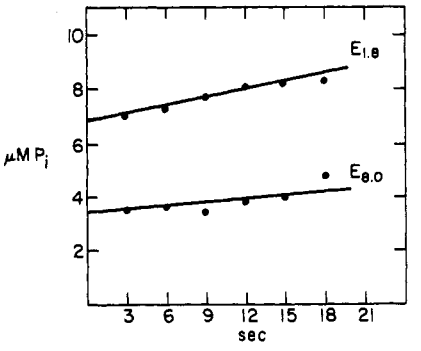


FIGURE 2: Time course of the ATPase reaction catalyzed by glutamine synthetase using enzyme of two adenylylation states ($E_{\langle 1.8\rangle}$ and $E_{\langle 8.0\rangle}$). The reaction mixtures contained 0.5 mM [γ-^{32}P]ATP, 20 mM glutamate, 10 mM $MgCl_2$, and glutamine synthetase (13.6 μM $E_{\langle 1.8\rangle}$ and 20.2 μM $E_{\langle 8.0\rangle}$). Additional details are given under Experimental Procedures.

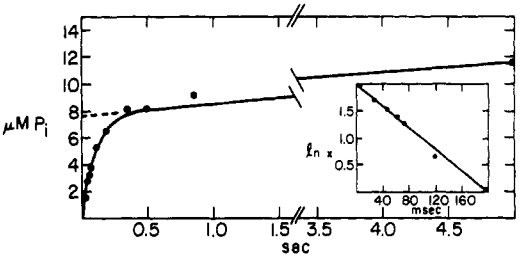


FIGURE 3: Time course of the ATPase reaction between 0.03 and 5 s. The reaction mixture contained 11.4 μM glutamine synthetase, 0.5 mM [γ-^{32}P]ATP, 20 mM glutamate, and 10 mM $MgCl_2$. The insert is a semilogarithmic plot of the difference between the individual time points of the extrapolated steady-state rate and the transient phase.

approximately equal for both forms of the enzyme: $\beta/(E_{\langle 0\rangle})$ = 0.59 for $E_{\langle 1.8\rangle}$ and 0.50 for $E_{\langle 8.0\rangle}$. Thus, only unadenylylated subunits are capable of catalyzing the formation of an acid-labile phosphate intermediate in the presence of Mg^{2+}. This finding agrees with the results of Rhee et al. (1976).

The transient phase of this pre-steady-state burst is shown in Figure 3. The steady-state rate constant (k_{ss}) taken from the "horizontal" part of the curve (t > 0.2 s) is equal to 0.078 s^{-1}, which agrees with corresponding rates obtained from spectrophotometric assays. The burst height (β = 7.6 μM P_i) is equivalent to 67% of the total unadenylylated subunit concentration. The semilogarithmic plot (inset) of the difference between the extrapolated steady-state points and experimental points vs. time shows that the transient phase can be adequately described by a single exponential. The transient rate constant (λ), evaluated from the slope of this plot and from data fitting to eq 8, equals 10.3 ± 0.2 s^{-1}.

(*B*) *Biosynthetic Reaction.* The time course of the biosynthetic reaction (at 24 °C) was determined in the range 0.03–0.9 s. The data clearly showed a burst of acid-labile phosphate, but no perceptible transient phase. The formation of P_i was linear from 0.03 to 0.9 s with a slope, k_{ss} = 11.4 s^{-1}, which agrees with rates from spectrophotometric assays. However, at 10 °C (Figure 4A), both the transient and steady-state phases are observed at reaction times less than 100 ms. The burst height, 7.0 μM phosphate, is equivalent to 57% of the total unadenylylated subunit concentration. The steady-state rate constant was calculated from additional data points taken at longer reaction times and was equal to 5.7 s^{-1}. The transient rate constant for the single exponential phase, as evaluated from data fitting to eq 8, equals 88 ± 10 s^{-1}. Under identical experimental conditions, the time course of the biosynthetic reaction was also followed by measuring

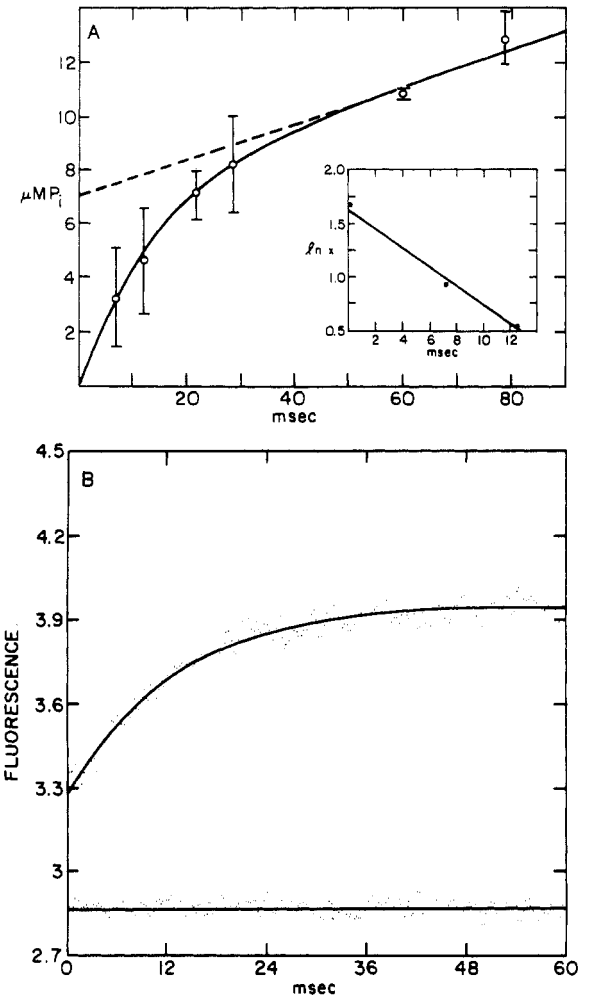


FIGURE 4: Rapid-quench and stopped-flow studies of the biosynthetic reaction at 10 °C. The reaction mixture contained 12.2 μM glutamine synthetase, 5 mM ATP, 20 mM glutamate, 10 mM NH_4Cl, and 15 mM $MgCl_2$. (A) Time course between 7.3 and 80 ms of the formation of acid-labile phosphate. The inset is a semilogarithmic plot of the difference between the individual time points of the extrapolated steady-state rate and the transient phase. (B) Time course between 2 and 60 ms of increasing protein fluorescence intensity at 334 nm. On a full time scale of 60 ms, 200 data points are acquired, the curve drawn through these points is a result of data fitting to a single exponential function. The base line is the protein fluorescence observed in the absence of substrates. The rate constant for the transient phase is 98 s^{-1}. Fluorescence intensities are in arbitrary units.

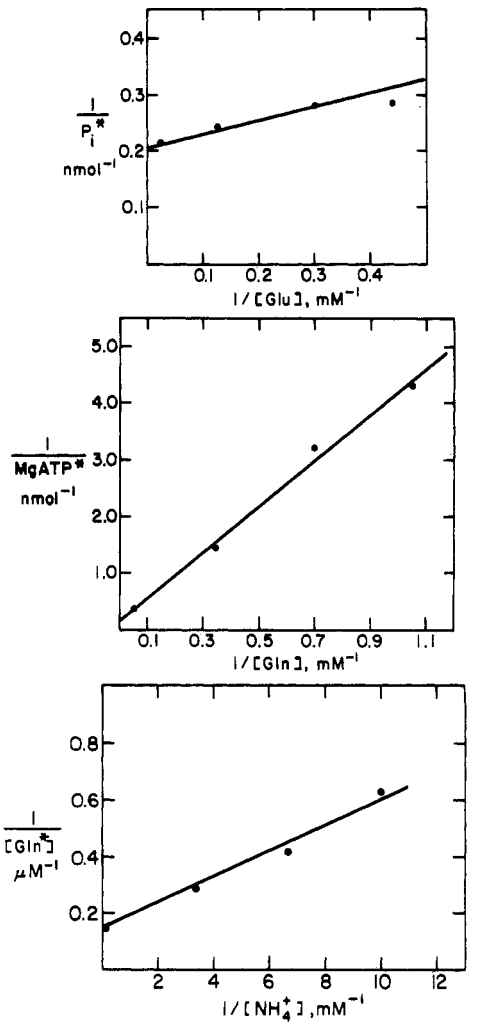


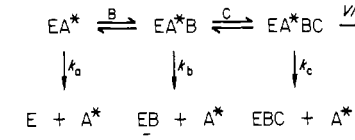
FIGURE 5: (Top) Isotope trapping of MgATP in the binary E–MgATP complex. As shown in the double-reciprocal plot, between 3.5 and 4.7 nmol of E–MgATP* is trapped as P_i* as the glutamate concentration is raised from 2.3 to 48.0 mM. From the intercept and slope of the plot, $P_i^*{}_{max}$ = 4.7 nmol and K'_{Glu} = 0.9 mM. Additional experimental details are described in the text. (Bottom) Isotope trapping of glutamate in the ternary E–MgATP–Glu complex. As the level of NH_4^+ is varied from 0.1 to 50.0 mM, 1.65–6.8 μM Gln* is formed from trapping of E–MgATP–Glu*. From the slope and intercept of the double-reciprocal plot, $Gln^*{}_{max}$ = 6.8 μM and $K'_{NH_4^+}$ = 0.29 mM. Other experimental details are described in the text. (Middle) Isotope trapping of MgADP in the binary E–MgADP complex. As glutamine levels are raised from 0.95 to 19.0 mM, 0.23–2.6 nmol of MgATP* is formed by trapping of E–MgADP*. From the slope and intercept of the double-reciprocal plot, $MgATP^*{}_{max}$ = 5.5 nmol and K'_{Gln} = 21 mM. Additional details are given in the text.

changes in protein fluorescence intensity at reaction times of 60 ms and less (Figure 4B). Following a small and very rapid initial increase in fluorescence intensity ($t_{1/2} \lesssim 1$ ms), a transient phase was observed in which the fluorescence intensity increased until a plateau was reached after about 40 ms. This transient phase was fitted to a single exponential function described by the curve drawn through the data points, giving a transient rate constant of 96 ± 10 s^{-1} (10 measurements) which is in good agreement with the value obtained from the rapid-quench experiment. An additional exponential is observed at $t > 200$ ms which corresponds to the release of MgADP.

Isotope Partitioning Experiments. A solution containing 4.6 nmol of E–MgATP* [(E–MgATP*) = $E_t/[1 + K_{i(MgATP)}/(MgATP^*)]$] was added to a solution containing a 500-fold excess of unlabeled ATP, a fixed level of NH_4^+, and variable levels of glutamate, and the reaction was quenched after 3 s. Shown in Figure 5 (top) is a double-reciprocal plot of nanomoles of P_i* formed vs. the corresponding concentration of glutamate. When the data are fitted to eq 7, the amount of P_i* formed at infinite glutamate ($P_i^*{}_{max}$) equals 4.7 ± 0.1 nmol. Thus, all of E–MgATP* is trapped as P_i* at infinite glutamate. The apparent Michaelis constant (K'_{Glu}) is 0.9 ± 0.2 mM. When the fixed level of NH_4^+ was changed (0.2–1.0 mM), the resulting double-reciprocal plots were, within experimental error, indistinguishable from that of Figure 5 (top), making it impossible to construct a pattern of NH_4^+ vs. Glu. Therefore, 0.9 mM is a limiting value of K'_{Glu} (see Appendix).

A solution containing 11.2 nmol of enzyme and 5 mM [^{14}C]glutamate was added to a 1000-fold excess of unlabeled glutamate and saturating levels of MgATP and NH_4^+. On the basis of a dissociation constant of 20 mM for E–Glu as determined by fluorometric titration (Timmons et al., 1974), the amount of E–Glu* present in the reaction was 2.2 nmol. After termination of the reaction (3 s) and product analysis, it was found that none of the E–Glu* was trapped as Gln*.

Scheme I



The results of a similar experiment with rapidly formed E–MgATP–Glu* are shown in Figure 5 (bottom). A solution containing 7.0 μM E–MgATP–Glu* [(E–MgATP–Glu*) = $E_t/[[1 + K_{i(MgATP)}/(MgATP)][1 + K_{i(Glu)}/(Glu^*)]]$] was diluted into a volume containing a 40-fold excess of unlabeled glutamate, a saturating fixed level of MgATP, and varying levels of NH_4^+, and the reaction was terminated after 5 s. From the double-reciprocal plot, Gln^*_{max} is 6.76 ± 0.05 μM, which, within experimental error, equals the concentration of E–MgATP–Glu*. Thus, all of the ternary complex is trapped as Gln* at infinite NH_4^+. $K'_{NH_4^+}$ = 0.290 ± 0.009 mM.

Results of isotope partitioning in the reverse biosynthetic reaction are shown in Figure 5 (middle). E–MgADP* (5.6 nmol) [(E–MgADP*) = $E_t/[1 + K_{i(MgADP)}/(MgADP^*)]$] is chased with a 250-fold excess of unlabeled MgADP, a saturating fixed level of phosphate, and varying levels of glutamine. When the results are plotted, $MgATP^*_{max}$ = 5.5 ± 0.4 nmol, indicating total trapping of E–MgADP* at infinite glutamine. K'_{Gln} = 21 ± 2 mM which, at saturating phosphate, should be very close to its limiting value.

Discussion

Two experimental approaches, rapid-quench kinetics and isotope partitioning kinetics, were applied to the study of the ATPase and biosynthetic reactions of *E. coli* glutamine synthetase. From rapid-quench data, the nature of the chemical intermediates involved in both reactions is determined, as are the rate constants for the interconversion steps of their respective enzyme–substrate complexes. From isotope partitioning data, the various Michaelis complexes that are of catalytic importance to the biosynthetic reaction are identified, and the rate constants for substrate desorption from these complexes are ascertained. These data when combined with our previously published steady-state kinetic experiments (Meek & Villafranca, 1980) provide a comprehensive mechanism for the reactions catalyzed by glutamine synthetase. The critical conclusion from the present study is that the previously proposed intermediate, γ-glutamyl phosphate, is the kinetically competent intermediate in the catalytic mechanism.

Isotope Partitioning Studies. The isotope trapping technique is a very useful tool for determining the relative rates of desorption of substrates from their respective Michaelis complexes. The method becomes a fully quantitative one when experimental results are plotted in a double-reciprocal manner (Cleland, 1975; Rose, 1980) from which P^*_{max} and K'_m values can be related to rate constants for substrate release. In the case of a terreactant enzyme (see Appendix), Scheme I shows that A* may dissociate from any of three transitory complexes in lieu of its conversion to P*. When A* is very sticky in both the EA*B and EA*BC complexes, k_b and $k_c \ll V/E_t$ and P^*_{max} = EA*. In this circumstance, eq 9 is valid (see Appendix)

$$K'_m/K_m = k_a/(V/E_t) \quad (9)$$

where K'_m and K_m are the limiting values of the Michaelis constants for the isotope partitioning data and steady-state kinetics, respectively.

For isotope partitioning of E–MgATP* (in Scheme I, EA* = E–MgATP*, B = Glu, and C = NH_4^+), double-reciprocal plots of $1/P_i^*$ vs. 1/Glu at several subsaturating levels of NH_4^+ consisted of a family of lines that were virtually superimposable, in which $P_i^*{}_{max}$ = E–MgATP* and K'_{Glu} = 0.9 ± 0.2 mM. Thus, MgATP is very sticky in both the E–MgATP–Glu and E–MgATP–Glu–NH_4^+ complexes (k_b and $k_c \ll V_1/E_t$).[6] This is the predicted result since the kinetic mechanism is a predominantly ordered one in which MgATP binds to free enzyme and should not readily desorb from the ternary and quaternary complexes (Meek & Villafranca, 1980). It should be emphasized, however, that a single turnover experiment lacks the sensitivity of a method such as isotope exchange for detecting small contributions from alternate pathways. By use of eq 9 and K_{Glu} = 4.5 ± 0.3 mM, $k_a/(V_1/E_t)$ = 0.2 ± 0.06, which upon substitution of V_1/E_t (14.5 ± 0.1 s^{-1}) yields the rate constant for MgATP release from E–MgATP, which for Schemes III and V, is k_2. From the analysis, $k_a = k_2$ = 2.9 ± 0.9 s^{-1}. Although MgATP is quite sticky in the E–MgATP complex, its rate of release is nearly twice V_2/E_t (1.65 s^{-1}), and so MgATP release is not solely rate limiting in the reverse biosynthetic reaction.

For the isotope partitioning of glutamate in E–MgATP–Glu* [in Scheme I, A* is unlabeled MgATP, B is Glu*, and when $k_c \ll V_1/E_t$, $K'_c/K_c = k_b/(V_1/E_t)$], Gln^*_{max} = E–MgATP–Glu* and $K'_{NH_4^+}$ = 0.29 ± 0.009 mM. Since all of the E–MgATP–Glu* complex is trapped at infinite NH_4^+, glutamate is extremely sticky in the quaternary E–MgATP–Glu–NH_4^+ complex ($k_c \ll V_1/E_t$), and when $K_{NH_4^+}$ = 0.22 ± 0.01 mM is used in the ratio of Michaelis constants, $k_b/(V_1/E_t)$ = 1.3 ± 0.1. Glutamate release from E–MgATP–Glu is only slightly faster than enzyme turnover: in Schemes III and V, k_4 = 19 ± 1.6 s^{-1}. Again, these results are in accord with a predominantly ordered mechanism. It should be reiterated here that we have measured glutamate release from the ternary E–MgATP–Glu complex that has not undergone significant conversion to E–MgADP–Glu~P, a complex from which glutamate cannot dissociate. As will be shown below, the unimolecular rate constant for conversion of E–MgATP–Glu to E–MgADP–Glu~P is 6.9 s^{-1} ($t_{1/2}$ = 0.1 s), so that after 8.5 ms only 6% of the reactants are present as E–MgADP–Glu~P when trapping with NH_4^+ occurs.

No Glu* could be trapped in the binary E–Glu* complex (in Scheme I, A* = Glu*, B = MgATP, and C = NH_4^+). Since glutamate is infinitely sticky in E–MgATP–Glu–NH_4^+ and is released from E–MgATP–Glu at a rate slightly greater than V_1/E_t, the inability to trap any Glu* in the binary complex must mean that either glutamate desorbs very rapidly from E–Glu (in Scheme V, $k_a = k_{20} \gg V_1/E_t$) or that the other substrates cannot bind to the E–Glu complex in the proper orientation for reaction. In either event, E–Glu is not a significant transitory complex on the reaction pathway. Timmons and co-workers have found that no change in protein fluorescence occurs when glutamate is added to a solution containing Mg^{2+}–enzyme. However, upon the subsequent addition of ATP, a fluorescence level is reached which is equal to that which is attributable to the E–MgADP–Glu~P complex (Timmons et al., 1974). In view of the reluctance of the E–Glu complex to participate in the biosynthetic reaction, these observations may be the result of rapid dissociation of glutamate from enzyme, followed by the sequential binding of MgATP and glutamate to form E–MgATP–Glu, a "preferred" complex for catalysis based on the work reported in our paper.

[6] In Scheme I, contribution from the k_b step at any concentration of B will be precluded when the level of C is "infinite". At nonsaturating changing fixed levels of C where $k_c \ll V/E_t$, no intercept effect will be observed unless k_b is finite (see Appendix).

Scheme II

$$E + S \rightleftharpoons ES \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EP \xrightarrow{k_7} E + P$$

In the reverse biosynthetic reaction, all of the E–MgADP* complex is trapped as MgATP* at infinite glutamine and saturating phosphate (in Scheme I, EA* = E–MgADP*, B = Gln, and C = P_i). MgADP is therefore very sticky in E–MgADP–Gln–P_i ($k_c \ll V_2/E_t$). Because the concentration of phosphate in our experiment is $33K_m$, the value of k_b is uninterpretable (see footnote 6), but since the reverse reaction has a preferred order of substrate addition in which MgADP binds to free enzyme, k_b should be small, if not zero. K'_{Gln} = 21 ± 2 mM, and by use of the steady-state value of K_{Gln} = 2.5 ± 0.2 mM, $k_a/(V_2/E_t)$ = 8.4 ± 1.5. MgADP dissociates from E–MgADP 8 times faster than the rate of catalysis of the reverse biosynthetic reaction. Upon substitution of V_2/E_t (1.65 ± 0.28 s^{-1}), $k_a = k_{17}$ = 14 ± 5 s^{-1} (in Scheme V). This value is equal to V_1/E_t (14.5 s^{-1}), so MgADP release is rate limiting for the forward biosynthetic reaction. This conclusion is corroborated by the results of equilibrium isotope exchange experiments. In their studies, Wedler & Boyer (1972) found that the MgATP ⇌ MgADP exchange was slower than either the NH_3 ⇌ glutamine or the glutamate ⇌ glutamine exchanges, indicating that the rate of nucleotide release and not substrate interconversion is rate limiting for catalysis.

ATPase Reaction. The fact that a pre-steady-state burst of acid-labile phosphate is observed when MgATP and glutamate are rapidly combined with enzyme constitutes compelling evidence that γ-glutamyl phosphate is a kinetically competent intermediate of the ATPase reaction. The observation of a similar burst in rapid-quench studies of *E. coli* carbamoyl-phosphate synthetase has been attributed to the existence of an analogous enzyme-bound intermediate, carboxy phosphate (Raushel & Villafranca, 1979). An abbreviated reaction scheme is shown in Scheme II, where ES and EP are the E–MgATP–Glu and E–MgADP–Glu~P complexes, respectively, and P represents MgADP, inorganic phosphate, and pyrrolidonecarboxylate. Since substrate concentrations were saturating in our rapid-quench studies, the expressions shown in eq 10–12 are valid (Shafer et al., 1972).

$$V_1/E_t = \frac{k_5k_7}{k_5 + k_6 + k_7} \quad (10)$$

$$\text{transient burst amplitude} = \frac{k_5(k_5 + k_6)}{(k_5 + k_6 + k_7)^2} = \beta/E_t \quad (11)$$

$$\text{transient burst rate} = k_5 + k_6 + k_7 = \lambda \quad (12)$$

By use of the experimental values of V_1/E_t(ATPase) = 0.0114 ± 0.0003 s^{-1}, β/E_t = 0.67 ± 0.1, and λ = 10.3 ± 0.2 s^{-1}, the three rate constants are solved from the three equations: k_5 = 6.9 ± 0.3 s^{-1}, k_6 = 3.4 ± 0.2 s^{-1}, and k_7 = 0.017 ± 0.0013 s^{-1}. The net rate constant for the slow cyclization of γ-Glu-P and subsequent product release, k_7, is rate determining for the ATPase reaction. The ratio k_5/k_6 is the equilibrium constant for transfer of the γ-phosphoryl group in the central complexes (K_{int}), and the calculated value of 2.0 is very similar to those found for a number of kinases, including ones that catalyze phosphorylation of carboxylic substrates (Lawson & Veech, 1979; Nageswara Rao et al., 1979; Knowles, 1980).

The finding that phosphorylation of glutamate is readily reversible on the enzyme is substantiated by the results of Midelfort & Rose (1976). In the strict absence of NH_4^+, the reversible formation of a γ-glutamyl phosphate intermediate was unequivocally demonstrated by the measurement of a

Scheme III

$$E \underset{k_2}{\overset{k_1,\ MgATP}{\rightleftharpoons}} E\text{-}MgATP \underset{k_4}{\overset{k_3,\ Glu}{\rightleftharpoons}} E\text{-}MgATP\text{-}Glu \underset{k_6}{\overset{k_5}{\rightleftharpoons}}$$

$$E\text{-}MgADP\text{-}Glu\sim P \xrightarrow{k_7} E + MgADP + P_i + \text{pyrrolidonecarboxylate}$$

Scheme IV

$$E\text{-}MgATP\text{-}Glu\text{-}NH_4^+ \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} E\text{-}MgADP\text{-}Glu\sim P\text{-}NH_4^+ \underset{k_{14}}{\overset{k_{13}}{\rightleftharpoons}}$$

$$E\text{-}MgADP\text{-}Gln\text{-}P_i \xrightarrow{k_{15}} E + P_i + Gln + MgADP$$

significant rate of βγ bridge/β nonbridge exchange of ^{18}O in βγ bridge labeled [^{18}O]ATP, which occurred only in the presence of glutamate. In our own studies we have measured a maximum rate of βγ bridge/β nonbridge positional oxygen exchange under conditions of the ATPase reaction: k_x = 1.8 s^{-1} (T. D. Meek, C. W. DeBrosse, and J. J. Villafranca, unpublished results). This maximum rate for positional isotope exchange is defined for the mechanism in Scheme III by eq 13 (T. D. Meek, C. W. DeBrosse, and J. J. Villafranca, unpublished results). By inserting the experimentally determined rate constants of k_4 = 19 s^{-1}, k_5 = 6.9 s^{-1}, k_6 = 3.4 s^{-1}, and k_7 = 0.017 s^{-1} into eq 13, we obtain a value of k_x = 1.7 s^{-1}, which is in excellent agreement with the experimental value of 1.8 s^{-1}.

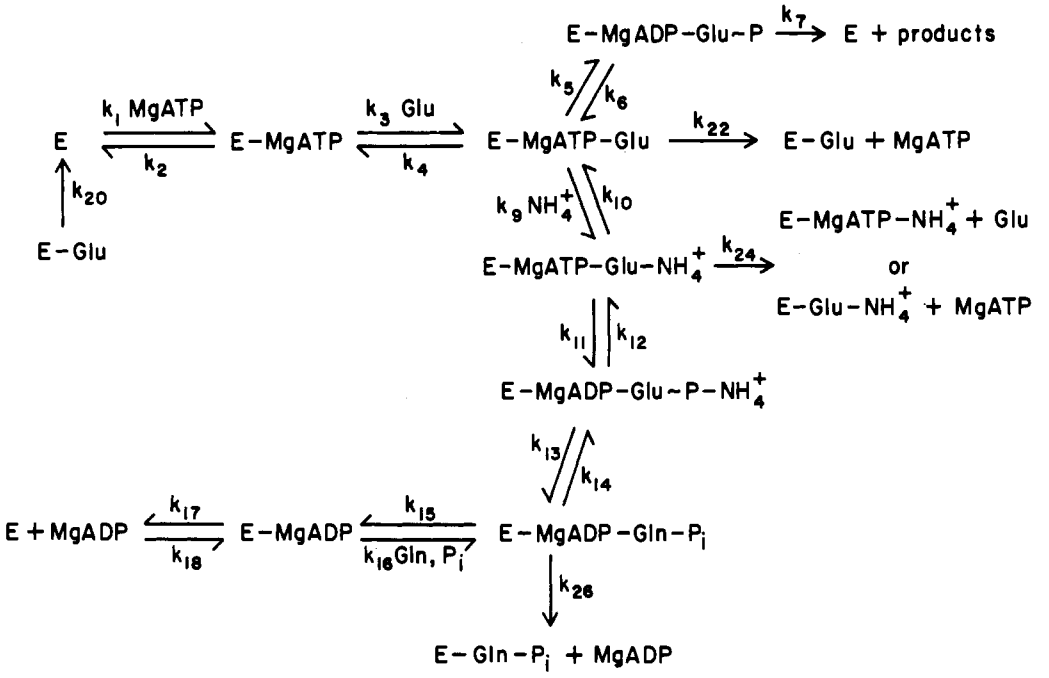
$$k_x = \frac{k_4k_5k_6}{(k_4 + k_5)(k_5 + k_6 + k_7)} \quad (13)$$

This result lends strong support to the proposal that the burst observed in the rapid-quench experiments is due solely to an E–MgADP–Glu~P complex. By design, our rapid-quench studies do not allow discrimination between acid-labile phosphate arising from E–MgADP–Glu~P or E–MgADP–P_i–pyrrolidonecarboxylate. However, since none of the [^{18}O]ATP that has undergone positional isotope exchange could have come from either an E–MgADP–P_i or an E–MgADP–P_i–pyrrolidonecarboxylate complex, agreement between the experimental and calculated values of k_x must mean that all of the phosphate formed in the burst phase is derived from E–MgADP–Glu~P.

Biosynthetic Reaction. The time course of the biosynthetic reaction is also characterized by a burst of acid-labile phosphate at 10 and 24 °C, as well as a rapid increase in protein fluorescence intensity at 10 °C. This rapid increase in protein fluorescence was first observed in another laboratory and attributed to the formation of enzyme-bound γ-glutamyl phosphate (Timmons et al., 1974; Rhee & Chock, 1976). Our study lends support to this conclusion. The transient rate constant for the protein fluorescence change is nearly equal to the transient burst rate from rapid-quench experiments, a rate which represents enzyme-catalyzed bond-breaking and bond-making steps (λ_{fluor} = 96 ± 10 s^{-1} and λ_{burst} = 88 ± 10 s^{-1}). This strongly suggests that the change in protein conformation either accompanies or directly effects the formation of a γ-glutamyl phosphate intermediate. It could be envisaged that the conformational change not only assists the transphosphorylation process by stabilizing nascent reaction intermediates but also protects the highly labile acyl phosphate from hydrolysis by situating this group in a cleft which excludes H_2O.

For the biosynthetic reaction, there is a minimum of three complexes which could account for the burst of acid-labile phosphate: E–MgADP–Glu~P, E–MgADP–Glu~P–NH_4^+, and E–MgADP–Gln–P_i. The correct complex cannot be E–MgADP–Glu~P because its formation from E–MgATP–

Scheme V



Glu proceeds with a rate constant (6.9 s^{-1}) that is slower than V_1/E_t for the biosynthetic reaction (14 s^{-1}). In view of this, the necessary rate requirements could be ascertained under conditions in which either of the other two complexes participate in the reaction: (1) NH_4^+ combines with E–MgATP–Glu prior to the phosphoryl transfer reaction, such that the resulting E–MgATP–Glu–NH_4^+ complex is converted to E–MgADP–Glu~P–NH_4^+ at a much faster rate than the analogous group transfer in the ATPase reaction ($k > 14$ s^{-1}); (2) the E–MgATP–Glu–NH_4^+ complex undergoes rapid conversion to E–MgADP–Gln–P_i by a totally concerted mechanism, followed by slow release of P_i from E–MgADP–Gln–P_i. For this second case, even though the ATPase reaction pathway must include a γ-glutamyl phosphate intermediate, it is entirely possible that the rapid intervention of NH_4^+ obviates formation of γ-glutamyl phosphate on the biosynthetic reaction pathway.

However, the possibility that E–MgADP–Gln–P_i is responsible for the pre-steady-state burst may be confidently ruled out on the basis of independent experimental evidence. Midelfort & Rose (1976) found that $\beta\gamma$ bridge/β nonbridge scrambling of $\beta\gamma$ bridge labeled [^{18}O]ATP occurred in the presence of saturating NH_4^+. The case of a concerted mechanism requires that the observed isotope scrambling arises solely from the return of the E–[β-^{18}O]MgADP–Gln–[$^{18}O_3$]P_i complex to [$^{18}O_4$]ATP. As Rose has pointed out, ATP that is resynthesized from this complex would eventually be depleted of ^{18}O in its γ-phosphoryl group because the three atoms of ^{18}O in the enzyme-bound P_i are randomly lost to the γ-carboxyl group of glutamate (Rose, 1979). Since no such "washout" was observed, and since it has also been shown that enzyme-bound P_i may freely rotate and lose its oxygen atoms to glutamate indiscriminately (Balakrishnan et al., 1978; Stokes & Boyer, 1976), P_i must be escaping from E–MgADP–Gln–P_i faster than the return of the complex to E–MgATP–Glu–NH_4^+, and the observed positional isotope exchange must involve a γ-glutamyl phosphate intermediate. Therefore, the correct intermediate is the E–MgADP–Glu~P–NH_4^+ complex.

The chemical events of the biosynthetic reaction are depicted in Scheme IV. On the basis of the arguments presented above, phosphate release from E–MgADP–Gln–P_i is much faster than the return of this complex to E–MgATP–Glu~P–NH_4^+, such that the net flux through the k_{13} step is irreversible under conditions of single enzymatic turnover (k_{13}, $k_{15} > k_{14}$). As a result, the rapid-quench data for the biosynthetic reaction are amenable to the same interpretation as that of the ATPase reaction. At 10 °C, $V_1/E_t = 4.0 \pm 0.4$ s^{-1}, $\beta/E_t = 0.57$, and $\lambda = 88 \pm 10$ s^{-1}. By use of eq 10–12 as before, $k_{11} = 54 \pm 6$ s^{-1}, $k_{12} = 28 \pm 8$ s^{-1}, and $k_{13} = 9.1 \pm 1.9$ s^{-1}. The slow step, k_{13}, represents a group of rate constants for all product release steps as well as the final chemical event, conversion of E–MgADP–Glu~P–NH_4^+ to E–MgADP–Gln–P_i.

At 10 °C, the equilibrium constant for phosphoryl transfer between E–MgATP–Glu–NH_4^+ and E–MgADP–Glu~P–NH_4^+ is comparable to values obtained for various kinases: $K_{int} = k_{11}/k_{12} = 1.9$. In view of the fact that a pre-steady-state burst of similar amplitude is observed at 24 °C, it is not unreasonable to suggest that K_{int} retains a value near 1.9 at that temperature, with concomitant increases in k_{11} and k_{12} (approximately 240 s^{-1} and 125 s^{-1}, respectively)[7] (given that, K_{int}(biosyn) and K_{int}(ATPase) would be nearly identical even though their respective values of k_f and k_r in the K_{int} expression differ by more than an order of magnitude). As such, a secondary role of NH_4^+ in the biosynthetic reaction would be to lower the activation energy for transphosphorylation, whether by a direct effect at the active site or by allosteric interaction. At either site, the binding of NH_4^+ would induce a series of conformational changes which act to stabilize the transition state(s) of phosphoryl group transfer. Bild & Boyer (1980) have recently shown that NH_4^+ binding to the *E. coli* enzyme promotes a cooperative effect among the subunits that results in increased catalysis.

The present data are summarized in Scheme V, where a combined pathway is shown for the ATPase and biosynthetic reactions of *E. coli* glutamine synthetase at pH 7.5. The

[7] These estimates are based on an extrapolated value of $\lambda_{25°C}$ taken from an Arrhenius plot of log λ vs. $1/T$. In addition, a second plot of log V_1/E_t vs. $1/T$ using a limited number of data points shows that the rate-limiting steps in the mechanism of the biosynthetic reaction do not change appreciably with temperature (10–37 °C).

complexes in this scheme do not reflect conformational changes in the enzyme and are assigned solely on the basis of relative rates of reaction as determined by chemical methods. However, as mentioned above, fluorescence studies have established that the biosynthetic and ATPase reactions are characterized by several changes in protein conformation (Timmons et al., 1974; Rhee & Chock, 1976). That the biosynthetic reaction proceeds with the preferred order of substrate addition and product release shown above is derived from previous steady-state kinetic studies (Meek & Villafranca, 1980), as well as the fact that those rate constants which represent the formation of abortive complexes integral to random pathways are insignificant: $k_{20} \gg V_1/E_t$; k_{22}, k_{24}, and $k_{26} \ll V_1/E_t$.

For the biosynthetic reaction, $k_2 = 2.9\ s^{-1}$ and $k_4 = 19\ s^{-1}$, and by use of these results with $K_{i(MgATP)}$ and $K_{i(Glu)}$ (given under Results), k_1 and k_3 can be calculated: $k_1 = 1.2 \times 10^4\ M^{-1}\ s^{-1}$ and $k_3 = 0.38 \times 10^4\ M^{-1}\ s^{-1}$. These values are much lower than those of typical substrate-association rate constants and several orders of magnitude lower than the rate of diffusion-controlled processes. This may be a result of the well-documented conformational changes that follow the sequential binding of MgATP and glutamate (Timmons et al., 1974). The calculation of k_{18} from k_{17} ($14\ s^{-1}$) and $K_{i(MgADP)}$ shows that MgADP has a much higher rate constant for binding: $k_{18} = 1.2 \times 10^5\ M^{-1}\ s^{-1}$.

For the ATPase reaction pathway, $k_5 = 6.9\ s^{-1}$, $k_6 = 3.4\ s^{-1}$, and, the rate-limiting step, $k_7 = 0.017\ s^{-1}$. This k_7 step represents the slow cyclization of γ-glutamyl phosphate to pyrrolidonecarboxylate and P_i, which probably occurs concomitantly with the dissociation of the acyl phosphate from the enzyme surface (Meister, 1974). As discussed above, the binding of NH_4^+ precludes the formation of E–MgADP–Glu~P, so that $k_9(NH_4^+) \gg k_5$ and $k_{10} < k_9(NH_4^+)$. At 10 °C, $k_{11} = 54\ s^{-1}$ and $k_{12} = 28\ s^{-1}$, which approximately equal $240\ s^{-1}$ and $125\ s^{-1}$, respectively, at 25 °C. The rate constants for the final chemical step (E–MgADP–Glu~P–NH_4^+ → E–MgADP–Gln–P_i) and the sequential release of P_i and Gln are neither rate limiting nor readily reversible ($k_{13} > V_1/E_t$, k_{14}, $k_{15} > V_1/E_t$, k_{16}). Finally the rate-limiting step for the biosynthetic reaction is the release of the last product, MgADP: $k_{17} = V_1/E_t = 14\ s^{-1}$.

Acknowledgments

We are deeply indebted to Dr. Frank M. Raushel for his assistance with data analysis and other helpful discussions and to Dr. Stephen J. Benkovic and Dr. Jubrail Rahil for the use and operation of the Durrum Multi-Mixer apparatus.

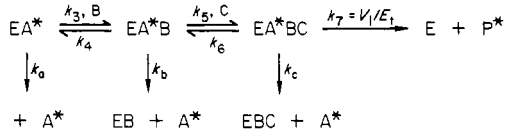
References

Balakrishnan, M. S., Sharp, T. R., & Villafranca, J. J. (1978) *Biochem. Biophys. Res. Commun. 85*, 991.
Benkovic, P. A., Bullard, W. P., de Maine, M. M., Fishbein, R., Schray, K. J., Steffens, J. J., & Benkovic, S. J. (1974) *J. Biol. Chem. 249*, 930.
Bild, G. S., & Boyer, P. D. (1980) *Biochemistry 19*, 5774.
Cleland, W. W. (1963) *Biochim. Biophys. Acta 67*, 104.
Cleland, W. W. (1967) *Adv. Enzymol. Relat. Areas Mol. Biol. 29*, 1.
Cleland, W. W. (1975) *Biochemistry 14*, 3220.
Dyson, R. D., & Isenberg, I. (1971) *Biochemistry 10*, 3233.
Gass, J. D., & Meister, A. (1970) *Biochemistry 9*, 842.
Ginsburg, A., Yeh, J., Hennig, S. B., & Denton, M. D. (1970) *Biochemistry 9*, 633.
Knowles, J. R. (1980) *Annu. Rev. Biochem. 49*, 877.
Krishnaswamy, P. R., Pamiljans, V., & Meister, A. (1962) *J. Biol. Chem. 237*, 2932.
Lawson, J. W. R., & Veech, R. L. (1979) *J. Biol. Chem. 254*, 6528.
Levitzki, A. (1970) *Anal. Biochem. 33*, 335.
Lowry, O. H., Rosebraugh, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 235.
Lymn, R. W., & Taylor, E. W. (1970) *Biochemistry 9*, 2975.
Meek, T. D., & Villafranca, J. J. (1980) *Biochemistry 19*, 5513.
Meek, T. D., Villafranca, J. J., & Mumma, R. O. (1981) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 40*, 1481.
Meister, A. (1974) *Enzymes, 3rd Ed. 10*, 699.
Midelfort, C. F., & Rose, I. A. (1976) *J. Biol. Chem. 251*, 5881.
Nageswara Rao, B. D., Kayne, F. J., & Cohn, M. (1979) *J. Biol. Chem. 254*, 2689.
Pamiljans, V., Krishnaswamy, P. R., Dumville, G., & Meister, A. (1962) *Biochemistry 1*, 153.
Prusiner, S., & Milner, L. (1970) *Anal. Biochem. 37*, 429.
Raushel, F. M., & Cleland, W. W. (1977) *Biochemistry 16*, 2176–2181.
Raushel, F. M., & Villafranca, J. J. (1979) *Biochemistry 18*, 3424.
Rhee, S. G., & Chock, P. B. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 476.
Rhee, S. G., Chock, P. B., & Stadtman, E. R. (1976) *Biochimie 58*, 35.
Rose, I. A. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol. 50*, 361.
Rose, I. A. (1980) *Methods Enzymol. 64*, 47.
Rose, I. A., O'Connell, E. L., Litwin, W., & BarTana, J. (1974) *J. Biol. Chem. 249*, 5163.
Schendel, P. F., & Wells, R. D. (1973) *J. Biol. Chem. 248*, 8319.
Shafer, J. A., Chiancone, E., Yielding, K. L., & Antonini, E. (1972) *Eur. J. Biochem. 28*, 528.
Shapiro, B. M., & Stadtman, E. R. (1970) *Methods Enzymol. 17A*, 910.
Stadtman, E. R., & Ginsburg, A. (1974) *Enzymes, 3rd Ed. 10*, 755.
Stokes, B. O., & Boyer, P. D. (1976) *J. Biol. Chem. 251*, 5558.
Timmons, R. B., Rhee, S. G., Luterman, D. L., & Chock, P. B. (1974) *Biochemistry 13*, 4479.
Todhunter, J. A., & Purich, D. L. (1975) *J. Biol. Chem. 250*, 3505.
Tsuda, Y., Stephani, R. A., & Meister, A. (1971) *Biochemistry 10*, 3186.
Wedler, F. C. (1974) *J. Biol. Chem. 249*, 5080.
Wedler, F. C., & Boyer, P. D. (1972) *J. Biol. Chem. 247*, 984.
Weisbrod, R., & Meister, A. (1973) *J. Biol. Chem. 248*, 3997.
Woolfolk, C. A., Shapiro, B. M., & Stadtman, E. R. (1966) *Arch. Biochem. Biophys. 116*, 177.

Appendix: Derivation of Equations for Isotope Partitioning Experiments for a Terreactant Enzyme

Pulse–chase or isotope partitioning experiments are the methods of choice for determining the dissociation rate of labeled substrate from binary or ternary complexes relative to V/E_t, the apparent net rate constant for one enzymic turnover. Several authors (Rose et al., 1974; Cleland, 1975; Rose, 1980) have derived expressions applicable for bireactant enzymes and have demonstrated that the method is useful for computing individual rate constants when sufficient data are gathered. Experiments are conducted at fixed levels of radiolabeled substrate while the other substrate is varied, and the data are plotted in a double-reciprocal manner. The method is applicable to random mechanisms with any number

of substrates and also to the first substrate in an ordered mechanism with two substrates. For an enzyme with three substrates and an ordered mechanism, the method can be applied by using radiolabel in the first or second substrate. The following derivation is for a terreactant enzyme:



The above scheme was used to analyze isotope partitioning data for glutamine synthetase in the main body of this paper. In a terreactant enzyme, A*, the radiolabeled compound in the pulse solution, may dissociate from any of the three transitory complexes EA*, EA*B, and EA*BC in the pulse–chase mixture in lieu of its conversion to P*.

The amount of P* formed from EA* can be represented by

$$\frac{P^*}{EA^*} = \left(\frac{EA^*B}{EA^*}\right)\left(\frac{EA^*BC}{EA^*B}\right)\left(\frac{P^*}{EA^*BC}\right) \quad (A1)$$

and each of the ratios may be expressed in terms of that fraction of enzyme complex which proceeds to P*; thus

$$\frac{P^*}{EA^*} = \left(\frac{k_3'}{k_a + k_3'}\right)\left(\frac{k_5'}{k_b + k_5'}\right)\left(\frac{k_7'}{k_c + k_7'}\right) \quad (A2)$$

where the primed constants represent the net rate constants for each step using the procedure of Cleland (1975). The expressions for each net rate constant are given in eq A3–A5.

$$k_7' = V_1/E_t \quad (A3)$$

$$k_5' = \frac{k_5(k_7 + k_c)[C]}{k_6 + k_7 + k_c} \quad (A4)$$

$$k_3' = \frac{k_3(k_5' + k_b)[B]}{k_4 + k_5' + k_b} = \frac{k_3\,[k_5(k_7 + k_c)[C] + k_b(k_6 + k_7 + k_c)][B]}{(k_4 + k_b)(k_6 + k_7 + k_c) + k_5(k_7 + k_c)[C]} \quad (A5)$$

Upon substitution and rearrangement, eq A2 becomes

$$\frac{EA^*}{P^*} = \left(\frac{k_7 + k_c}{k_7}\right)\left[\frac{k_b(k_6 + k_7 + k_c)}{k_5(k_7 + k_c)[C]} + 1\right] \times \left[\frac{k_a[(k_4 + k_b)(k_6 + k_7 + k_c) + k_5(k_7 + k_c)[C]]}{k_3[B][k_5(k_7 + k_c)[C] + k_b(k_6 + k_7 + k_c)]} + 1\right] \quad (A6)$$

Equation A6 is applicable to random or ordered reaction mechanisms.

It is informative to examine several limiting cases of eq A6 and their consequences in terms of calculating individual rate constants for the terreactant scheme depicted above. When C is saturating, i.e., [C] $\rightarrow \infty$, all contributions from k_b are precluded.

For an ordered reaction mechanism with k_b, $k_c = 0$, then eq A6 reduces to

$$\frac{EA^*}{P^*} = \frac{K'_b}{[B]}\left(\frac{K'_c}{[C]} + 1\right) + 1 \quad (A7)$$

where $K'_b = k_a/k_3$ and $K'_c = k_4(k_6 + k_7)(k_5k_7)$. Thus, a plot of $1/P^*$ vs. $1/[B]$ at various levels of C would result in a family of lines with $EA^* = P^*_{max}$ when [C] $\rightarrow \infty$ and [B] $\rightarrow \infty$. If the family of lines do not intercept the $1/P^*$ axis at a common point, then either $k_b \neq 0$ or $k_c \neq 0$, or both. This latter result is expected for a random mechanism. If $k_b \neq 0$ and $k_c = 0$, i.e., the first two substrates are random and the third ordered, limits can be placed on k_a and k_b from replots of slopes and intercepts vs. $1/[C]$. For a fully ordered mechanism, replots of the intercept on the $1/[B]$ axis vs. $1/[C]$ will provide a value for K'_c.

Combining pulse–chase experiments with steady-state kinetic measurements permits evaluation of k_a as follows. From steady-state kinetics

$$\frac{V}{K_b} = k_3'[EA] = k_3'E_t \text{ at } [A] \rightarrow \infty \quad (A8)$$

and

$$\frac{K_b}{V} = \frac{1}{E_t}\left[\frac{k_4(k_6 + k_7)}{k_3k_5k_7[C]} + \frac{1}{k_3}\right] \quad (A9)$$

which reduces to eq 10 at [C] $\rightarrow \infty$.

$$\frac{K_b}{V} = \frac{1}{k_3E_t} \quad (A10)$$

Combining eq A10 with $K'_b = k_a/k_3$, one arrives at the expression

$$\frac{K'_b}{K_b} = \frac{k_a}{V/E_t} \quad (A11)$$

which permits calculation of k_a or at least limits on the ratio of k_a to V/E_t. Equation A11 is identical in form with eq 9 in the main body of this paper.

For the case where radiolabel is in B with a terreactant enzyme, the experimental consequences are the same as those for a two-substrate enzyme (Cleland, 1975; Rose, 1980).

References

Cleland, W. W. (1975) *Biochemistry 14*, 3220.
Rose, I. A. (1980) *Methods Enzymol. 64*, 47.
Rose, I. A., O'Connell, E. L., Litwin, W., & BarTana, J. (1974) *J. Biol. Chem. 249*, 5163.