Subunit Interaction during Catalysis: Ammonium Ion Modulation of Catalytic Steps in the *Escherichia coli* Glutamine Synthetase Reaction†

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Appendix: Theoretical Relationships for Oxygen Transfer Catalyzed by *Escherichia coli* Glutamine Synthetase during Net Adenosine 5′-Triphosphate Cleavage

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ABSTRACT: A new approach for assessing if catalytic cooperativity may occur between subunits has been applied to *Escherichia coli* glutamine synthetase. The extent of oxygen exchange between bound [^{18}O]glutamate and phosphate per molecule of glutamine formed was evaluated at various NH_4^+ concentrations. This allows calculation of the minimum number of reaction reversals in which bound glutamine is converted to bound glutamate prior to release of glutamine. At 1000 μM NH_4^+ no detectable reversals occurred, and only one glutamate oxygen appeared in product phosphate as required by the reaction mechanism. However, at 10 μM NH_4^+ over 15 reversals of bound glutamine formation occurred. Controls showed that under the experimental conditions free glutamine does not become significantly involved in exchange and, therefore, the reversal of the oxygen exchange steps appears to be limited to bound glutamine. In contrast to the effect seen with NH_4^+, adenosine 5′-triphosphate concentration appears to modulate the exchange of oxygen between glutamate and phosphate only slightly. These findings are interpreted as showing that NH_4^+ either promotes the dissociation of one of the reaction products or decreases the participation of bound products in the exchange. The NH_4^+ modulation of the oxygen exchange is consistent with binding of NH_4^+ at one catalytic site promoting catalytic events at an alternate catalytic site but does not eliminate all other explanations.

Alternating site cooperativity occurs when two or more identical catalytic sites of a multisubunit enzyme participate in sequence in a manner such that acceleration of catalytic events at one site results from binding of substrate(s) at an alternate site. Coordination between subunits of enzymes showing such catalytic cooperativity would be achieved through conformational interactions. The advantage an enzyme might gain from alternating site cooperativity is that energy of binding of a substrate to one subunit may increase flux through rate-limiting steps on an alternate subunit and thereby increase catalytic efficiency. This will result in a dependency of intermediate reaction steps, subsequent to substrate binding, on substrate concentration. Such modulation of intermediate catalytic steps by substrate offers a potential way of detecting catalytic cooperativity. Results of this approach with *Escherichia coli* glutamine synthetase are given in this paper.

Subunit catalytic cooperativity has been considered for a number of enzymes (Harada & Wolfe, 1968; Lazdunski, 1974; Fersht, 1975; Adolfsen & Moudrianakis, 1976; Levitzki & Koshland, 1976). However, convincing evidence has been difficult to obtain. A new approach for detection of substrate modulation of intermediate reaction steps, which involves the measurement of the extent of an oxygen-exchange reaction at high and low substrate concentrations, has been applied to enzymic synthesis and hydrolysis of adenosine 5′-triphosphate (ATP) (Hackney & Boyer, 1978; Hutton & Boyer, 1979). During ATP hydrolysis by mitochondrial ATPase the extent of water oxygen incorporated into each inorganic phosphate (P_i) formed (intermediate $P_i \rightleftharpoons HOH$ exchange) increased markedly as ATP concentration was lowered. This was shown to result from an increase in the number of reversals of formation of bound adenosine 5′-diphosphate (ADP) and P_i from bound ATP per mole of ATP cleaved. A similar approach is also applicable to enzymes with the potential for catalyzing exchange of equivalent oxygens in carboxylate groups and was recently applied to *E. coli* succinyl-CoA synthetase by Bild et al. (1980). They showed that a pronounced increase occurs in the oxygen exchange between succinate and phosphate as the ATP concentration was lowered, consistent with catalytic cooperativity.

E. coli glutamine synthetase appears to lend itself well to this approach for assessment of catalytic cooperativity. The enzyme catalyzes a transfer of an oxygen from the γ-carboxyl group of glutamate to phosphate (Kowalsky et al., 1956; Boyer et al., 1956). Measurement of oxygen exchange involving phosphate or carboxyl groups can be particularly advantageous for detection of catalytic cooperativity since carboxyl or phosphate oxygens of bound substrates may participate equally in catalysis. Reversal of steps involved in oxygen transfer could lead to the presence of more than one exchanged oxygen in each molecule of product released. The extent of reversal of an exchange step prior to product release a high and low substrate concentrations can thus be detected.

E. coli glutamine synthetase (M_r = 600 000) (Shapiro & Ginsburg, 1968) is composed of 12 identical subunits arranged in two stacked eclipsed hexagons (Valentine et al., 1968; Woolfolk et al., 1966) and is subject to a multitude of feedback controls (Stadtman & Ginsburg, 1974). In addition, the enzyme is regulated by adenylylation–deadenylylation of a tyrosine residue in each of the subunits. Heterologous interactions between adenylylated and unadenylylated subunits in hybrid enzyme forms have been shown to affect catalytic and stability characteristics (Ginsburg et al., 1970). Homologous interactions, reflecting negative cooperativity of

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substrate binding, are observed during irreversible inhibition of unadenylylated glutamine synthetase by the *S* isomer of methionine sulfoximine in the presence of ATP·Mg (S. G. Rhee, P. B. Chock, F. C. Wedler, and Y. Sugiyama, unpublished experiments) and Mg·AMP–PNP (Wedler, 1980). Negative cooperativity of the reversible methionine sulfoximine binding is also observed (Shrake et al., 1980). Enzymes that show negative cooperativity of substrate binding are good candidates for catalytic cooperativity.

Experimental Procedures

Glutamine synthetase, purified by the Woolfolk method (Woolfolk et al., 1966), was obtained from Dr. David Eisenberg and was homogeneous by polyacrylamide gel electrophoresis. Preparations had an adenylylation state of 10 molecules of AMP bound/12 subunits. The specific activity of the enzyme in a γ-glutamyl transfer assay (Shapiro & Stadtman, 1970) was 54 μmol of product min^{-1} (mg of protein)$^{-1}$. Protein concentrations were determined by the method of Lowry et al. (1951) with bovine serum albumin as standard.

Lactate dehydrogenase (rabbit muscle) was obtained as an ammonium sulfate precipitate from Boehringer Mannheim. Pyruvate kinase (rabbit muscle) was obtained as the lyophilized salt-free powder from Sigma Chemical Co. Lactate dehydrogenase was freed from NH_4^+ ion by filtration twice through small Sephadex columns in a centrifuge (Penefsky, 1977).

[γ-^{32}P]ATP (>2000 Ci/mmol) was purchased from Amersham. Highly enriched [^{18}O]HOH for preparation of [^{18}O]glutamate and [^{18}O]KH_2PO_4 was purchased from Norsk Hydro, New York, NY 10022. [^{18}O]P_i (99% enriched) was prepared by hydrolysis of PCl_5 in enriched water by the method of Risley & Van Etten (1978) as modified by Hackney et al. (1980).

[^{18}O]Glutamate was prepared by equilibrating glutamic acid hydrochloride with highly enriched water in a sealed ampule at 120 °C for 48 h essentially as described by Kowalsky et al. (1956). The labeled water was recovered by vacuum transfer and the ^{18}O-labeled glutamate was reequilibrated with additional 99% H^{18}OH for another 48 h at 120 °C. Labeled water was again recovered by vacuum transfer and the ^{18}O-labeled glutamate was collected.

[γ-^{18}O]Glutamine was prepared in a reaction mixture containing 20 mM Hepes,[1] 70 mM KCl, 1 mM $MnCl_2$, 20 mM glutamine, 5 mM ADP, 20 mM 99% [^{18}O]KH_2PO_4, and 0.5 mg of glutamine synthetase in a final volume of 5.0 mL at 37 °C at pH 6.5 for 3 h. The reaction mixture was quenched by $CHCl_3$, diluted to 40 mL with distilled water to lower the ionic strength of the solution, and applied to a column of DEAE-cellulose (formate). Glutamine was separated from all other negatively charged substrates by elution with a linear gradient of 25 mL of distilled water and 25 mL of 1.0 M triethylammonium formate, pH 3.2. Glutamine was then separated from NH_4^+ and other cations by adjusting the pH to 10–11 with KOH and applying to a Dowex-2X(OH) column. The column was rinsed with distilled water, and glutamine was eluted with 1 M formic acid and lyophilized. For determination of the ^{18}O content of glutamine, 10 μmol was converted to CO_2 by heating under vacuum with guanidine hydrochloride as described previously (Boyer & Bryan, 1967). The average ^{18}O value of CO_2 produced by prolysis of glutamine has been found to be 0.756 of that expected on the basis of the assumption that all oxygens of the sample contributed equally to the CO_2 formed (Stokes & Boyer, 1976). There is, thus, a preferential conversion of the α-carboxyl oxygens to CO_2. Analysis by this method established the ^{18}O content of glutamine in the γ position to be 20.7 atom %.

The ^{18}O content of glutamate was determined by equilibrating the γ-carboxyl oxygens of glutamate with P_i through the glutamine synthetase reaction. The reaction mixture initially contained 20 mM imidazole, 70 mM KCl, 2.5 mM $MgCl_2$, 1 mM $MnCl_2$, 5.0 mM [^{18}O]glutamate, 5.0 mM NH_4Cl, 2.0 mM ATP, [γ-^{32}P]ATP (500 000 cpm), and 1.0 mg of glutamine synthetase in a total volume of 10.0 mL at pH 6.5. Samples were incubated under toluene to avoid microbial contamination at 37 °C for a period of 6 days. An additional 1.0 mg of enzyme was added after 2 and 4 days. A similar reaction mixture lacking glutamine synthetase was treated in an identical manner and served as a control and to correct for the decay of ^{32}P. The incubation time required to reach chemical equilibrium was determined by measuring the ^{32}P distribution between P_i and ATP over a period of time until the ratio was constant. After incubation for 100 and 200 times as long as required for reaching chemical equilibrium, the P_i concentration (Sumner, 1944) and ^{18}O content of P_i (Boyer & Bryan, 1967) were measured. For the latter, the P_i was diluted 50-fold to 200-fold with nonlabeled P_i. The ^{18}O content of both P_i samples was the same, giving assurance that isotopic equilibrium had been reached. An average value of 83.4 atom % excess ^{18}O of the γ-carboxyl group of the original glutamate was calculated from six separate analyses. This value agreed well with a value of 83.9 atom % excess obtained by the direct analysis of ^{18}O by conversion of glutamate to CO_2 essentially as described previously (Boyer & Bryan, 1967). The ^{18}O content of the two carboxyl groups of glutamate prepared in the manner described were thus labeled to an equal extent.

Reaction mixtures in the substrate modulation experiments were at 37 °C and contained 20 mM imidazole, 70 mM KCl, 1 mM $MnCl_2$, 20 mM $MgCl_2$, 10 mM [^{18}O]glutamate (84%), 1 mM ATP, 0.30 mM NADH, 2.5 mM PEP, 25 units of pyruvate kinase, 50 units of lactate dehydrogenase, 50 μg of glutamine synthetase, and varying amounts of NH_4Cl in a total volume of 2.0 mL at pH 6.5 in a quartz cuvette. The approximate progress of the reaction was monitored at 340 nm in a Cary 16 spectrophotometer. NH_4Cl was added to the cuvette at a constant, known rate through a Sage Instruments syringe pump Model 341A fitted with a 25-μL Hamilton syringe. The cuvette holder was fitted with an air-driven microstirrer (Hamilton et al., 1979) to ensure mixing. When examining the effect of ATP concentration on the $O_{glutamate} \rightleftharpoons O_{P_i}$ exchange, the NH_4^+ ion concentration was 1.0 mM. ATP was maintained at a constant, known concentration by the pyruvate kinase regenerating system. The reaction was quenched by mixing the solution with 2.0 mL of a suspension of charcoal (30 mg/mL) in 1 M $HClO_4$. The charcoal was removed by centrifugation. The phosphate concentration was determined (Sumner, 1944) both before and after addition of 10.0 μmol of carrier KH_2PO_4. Phosphate was isolated by extraction as the molybdate complex and analyzed for ^{18}O content by conversion to CO_2 with guanidine hydrochloride.

Results

Experimental Design. Understanding of the experimental design is aided by the schematic representation of the glutamine synthetase reaction with an ADP-trapping system shown in Figure 1. In net glutamine synthesis oxygen is transferred from the γ-carboxyl group of glutamate to the P_i formed from

[1] Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PEP, phosphoenolpyruvate; APP(NH)P, adenyl-5′-yl imidodiphosphate; DEAE, diethylaminoethyl; NADH, reduced nicotinamide adenine dinucleotide.

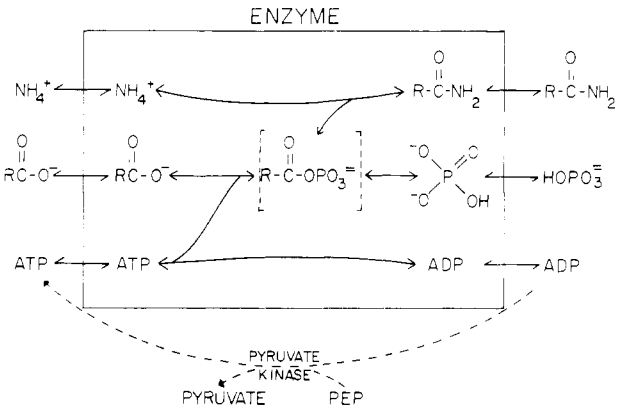

FIGURE 1: Schematic representation of the *E. coli* glutamine synthetase reaction depicting the ADP trap and ATP-regenerating system. R. is $HOCOCHNH_2CH_2CH_2^-$.

ATP (Kowalsky et al., 1956). With glutamine synthetase from *E. coli* the occurrence of oxygen exchange between enzyme-bound P_i and glutamate requires formation of ATP from the P_i.[2] Any exchange of oxygen between glutamate and bound P_i likely occurs prior to the release of ADP since medium ADP is rapidly converted to ATP by the pyruvate kinase trapping system. The γ-carboxyl group of bound glutamate is free to rotate in such a manner that either carboxylate oxygen can participate in the reaction (Stokes & Boyer, 1976). In addition, it has been shown that P_i rapidly rotates or tumbles while remaining bound to the enzyme so that all four phosphate oxygens have the potential to undergo exchange (Balakrishnan et al., 1978). Therefore, the possibility exists that both oxygens originating in a glutamate may appear in product phosphate if extensive reversal of the oxygen-exchange steps occurs prior to ADP release. Kowalsky et al. (1956) have shown that when all substrates are present at high concentrations there is a stoichiometric transfer of a single oxygen from glutamate to phosphate during net glutamine synthesis, thus implying there is no reversal of the oxygen-exchange steps. However, if subunit interactions occur such that the binding of a substrate to one subunit influences either a dissociation constant or an oxygen-transfer rate constant on a second subunit, then the potential exists that at low substrate concentrations more than one glutamyl oxygen may be transferred to phosphate during net glutamine synthesis. Thus, measurement of the extent of exchange of oxygen between glutamate and P_i at high and low substrate concentration provides an excellent probe for possible subunit catalytic cooperativity with *E. coli* glutamine synthetase.

Regulation of the Steady-State Concentration of NH_4^+. In those experiments in which the effect of NH_4^+ on the $^{18}O_{glu} \rightleftharpoons {}^{18}O_{P_i}$ exchange was examined in the 10–50 μM range, NH_4Cl was delivered at a constant rate through a Sage Instruments syringe pump to the reaction mixture in a stirred cuvette. For determination of the flow rate necessary to maintain a given NH_4^+ concentration, 30 mM NH_4Cl was delivered to a complete reaction mixture at various preset rate positions. The progress of the reaction was monitored by observing NADH oxidation via the pyruvate kinase–lactate dehydrogenase coupled assay system. The concentration of

[2] γ-Glutamyl phosphate is indicated as a likely phosphorylated intermediate in Figure 1. Reversible formation of γ-glutamyl phosphate from bound P_i and glutamine would not give any oxygen exchange. Reformation of bound ATP and of bound glutamate with the γ-carboxyl group of bound glutamate exhibiting torsional symmetry is necessary for oxygen exchange.

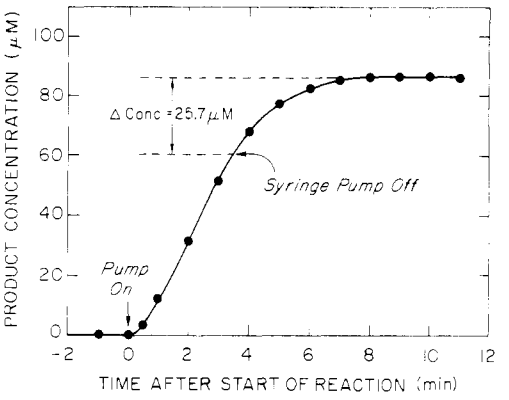

FIGURE 2: Calibration of syringe pump to maintain a steady-state level of NH_4^+. The reaction mixture is described under Experimental Procedures except that nonlabeled glutamate was used and no NH_4^+ was added initially. The syringe pump containing 30 mM NH_4Cl was turned on to start the reaction. The progress of the reaction was monitored spectrophotometrically at 340 nm. After a steady-state reaction velocity was obtained, the syringe pump was turned off and the amount of continued NADH disappearance determined. The data reported in this figure are for a flow rate of 2.75 μL/min and represent a steady-state concentration of 25 μM NH_4^+ in the reaction mixture.

Table I: Effect of NH_4^+ Concentration on $O_{glutamate} \rightleftharpoons O_{P_i}$ Exchange during Glutamine Formation Catalyzed by *E. coli* Glutamine Synthetase

$[NH_4^+]$ (μM)	$[^{18}O]P_i$ (atom %)[a]	$\bar{O}'$[b]	R[c]
1000	20.8	0.99	0
100	25.4	1.21	0.86
50	27.4	1.31	1.7
25	31.4	1.50	7
10	32.7	1.56	>15[d]

[a] Minimum possible calculated value is 21.0% and maximum possible value is 33.6% based on the original ^{18}O content of glutamate as 83.9 atom %. [b] $\bar{O}'$ is the average number of glutamate oxygens appearing in phosphate. [c] R is the estimated average number of reaction reversals which have occurred (the number of times bound glutamine forms bound glutamate before product dissociation stops exchange). [d] At high R values the error in the calculation of R becomes large. Due to the inherent inaccuracies in the method for determining the value of R at high $\bar{O}'$, values we conservatively state R as >15.

NH_4^+ in the reaction mixture at each of the syringe delivery rate positions was determined after attainment of a steady-state reaction velocity by measuring the amount of NADH oxidation which occurred after shutting off the syringe pump (Figure 2). Prior to initiating an exchange measurement, NH_4^+ was added to the reaction mixture at the desired steady-state concentration. The pump with the appropriate setting was turned on at the time of addition of glutamine synthetase.

This method for maintaining a steady-state concentration is applicable only when the substrate NH_4^+ concentration is considerably less than that required for maximum velocity. For experiments near 100 μM NH_4^+, the initial concentration of NH_4^+ in the reaction mixture was 120 μM, and the NH_4^+ concentration was maintained between 80 and 120 μM by monitoring the reaction spectrophotometrically and manually adding small aliquots of 30 mM NH_4Cl when necessary. In the range of 100 μM and higher, exchange and net reaction rates are relatively insensitive to NH_4^+ concentration; thus, the manual regulation was satisfactory. For measurement of the extent of exchange at 1000 μM NH_4^+, no additional NH_4^+ was added during the course of the reaction; thus, the actual concentration dropped from 1000 to ~700 μM at the end of the reaction.

Effect of NH_4^+ on $^{18}O_{glutamate} \rightleftharpoons O_{P_i}$ Exchange. This was measured by the extent of the transfer of glutamate oxygens to P_i during net synthesis of glutamine with initial NH_4^+ concentrations ranging from 10 to 1000 μM. The K_m value for NH_4^+ under our experimental conditions was $\sim$30 μM. As shown in Table I, at 1000 μM NH_4^+ the average number of glutamate oxygens present in P_i for each P_i produced, $\bar{O}'$, was nearly 1.0, in close agreement with that reported by Kowalsky et al. (1956). Thus, no apparent reversal of the oxygen-exchange steps occurred. However, at 10 μM ammonia an average of 1.56 glutamate oxygens were found in each phosphate produced. This effect of NH_4^+ concentration has been observed in two additional experimental series (data not shown) under the conditions used for the experiments in Table I.

Stokes & Boyer (1976) determined that at high substrate concentrations the rate of dissociation of glutamate is much slower than either the substrate interconversion or ADP release rates. With the assumption that the same relative rates hold at low NH_4^+ concentration, the maximum theoretical average number of glutamate oxygens transferred to each P_i would be 1.6; the original two oxygens of the γ-carboxyl group of glutamate at equilibrium distribute equally between the four phosphate oxygens and the amide oxygen of glutamine. Thus, each of the five product oxygens has a $^2/_5$ possibility of containing an oxygen derived from glutamate. Therefore, at equilibrium, phosphate should contain $4(^2/_5) = 1.6$ γ-glutamyl oxygens. The number of reversals occurring at 10 μM NH_4^+ was estimated from eq 1 (see Appendix) where R equals the

$$R = \frac{8\bar{O}' - 8}{8 - 5\bar{O}'} \quad (1)$$

number of reversals. A reasonable estimate is that at 10 μM NH_4^+ reversal of the oxygen-exchange reaction occurs over 15 times prior to the release of ADP.

The use of eq 1 is based on the assumption that the rate of glutamate release, shown by Wedler & Boyer (1972) to be slower than the rate of release of ADP, P_i, or glutamine at chemical equilibrium, remains slower than release of products at low NH_4^+ concentration. If the rate of glutamate release were comparable to the release of any product, eq 1 would overestimate the number of reversals. Conversely, if exchange were limited by the rate of rotation or tumbling of the γ-carboxyl group of bound glutamate or of the bound P_i, the equation wouuld underestimate the number of reversals. Irrespective of these considerations, the important point remains that as the NH_4^+ concentration is lowered, oxygen transfer from glutamate to each P_i formed is increased.

Effect of ATP on $^{18}O_{glutamate} \rightleftharpoons O_{P_i}$ Exchange. This was measured by the extent of the transfer of glutamate oxygens to phosphate during the net synthesis of glutamine at ATP concentrations ranging from 500 to 5 μM. The K_m for ATP under our experimental conditions was $\sim$40 μM. As shown in Table II, there is a slight increase in the amount of glutamate oxygen appearing in P_i during net glutamine synthesis as ATP concentration is lowered. However, the increase in oxygen exchange which occurs at 5 μM ATP, the lowest concentration examined, represents only 0.5 reversals of the oxygen-exchange steps prior to ADP release for each P_i synthesized. This is an appreciable effect, but not nearly as pronounced as we found for NH_4^+.

Medium $^{18}O_{glutamine} \rightleftharpoons O_{P_i}$ Exchange during Net Glutamine Synthesis. A possible alternate explanation of the NH_4^+ concentration dependent modulation of the $O_{glu} \rightleftharpoons O_{P_i}$ exchange is that NH_4^+ (or NH_3) and glutamine complete for a common binding site on glutamine synthetase such that high

Table II: Effect of ATP Concentration on $O_{glutamate} \rightleftharpoons O_{P_i}$ Exchange during Glutamine Formation Catalyzed by *E. coli* Glutamine Synthetase

[ATP] (μM)	$[^{18}O]P_i$[a] (atom %)	$\bar{O}'$[b]	R[c]
500	20.85	0.99	0
90	21.85	1.04	0.11
50	23.00	1.10	0.32
5	24.17	1.15	0.53

[a] Minimum possible calculated value is 21.0% and maximum possible value is 33.6% based on the original ^{18}O content of glutamate as 83.9 atom %. [b] $\bar{O}'$ is the average number of glutamate oxygens appearing in phosphate. [c] R is the estimated average number of reaction reversals which have occurred (the number of times bound glutamine forms bound glutamate before product dissociation stops exchange).

Table III: Medium $O_{glutamine} \rightleftharpoons O_{P_i}$ Exchange during Net Glutamine Synthesis at 1000 and 10 μM Ammonia[a]

$[NH_4^+]$ (μM)	P_i produced (nmol)	$[^{18}O]P_i$ (atom % excess)	max possible no. of gln oxygens in P_i for each P_i formed[b]
1000	600	0.071	0.04
10	600	0.212	0.11

[a] Reaction conditions were the same as described in Tables I and II (see Experimental Procedures) except that 200 μM (400 nmol) $[^{18}O]$glutamine (20.7 atom%) was added to the reaction mixture and glutamate contained natural abundance of ^{18}O. [b] Represents the upper limit of oxygen exchange based on the assumption that 1000 nmol of glutamine and 600 nmol of P_i were present during the entire reaction time.

$[NH_4^+]$ prevents glutamine released to the medium from rebinding and becoming reinvolved in oxygen exchange prior to the release of ADP, thus resulting in a more extensive loss of glutamate oxygens to phosphate at the lower NH_4^+ concentrations. This possibility needs to be considered since it has been shown that at high substrate concentrations dissociation of P_i and glutamine is more rapid than dissociation of ADP and that at 10 mM glutamine and 2 mM P_i exchange between P_i and glutamine is quite rapid (Stokes & Boyer, 1976). However, whether significant exchange occurs between medium glutamine and phosphate at their relatively low medium concentrations encountered during the course of these experiments needed to be investigated.

Four hundred nanomoles of glutamine labeled with ^{18}O exclusively in the amide oxygen was added to the standard reaction mixture to find whether glutamine, once released to the medium at the concentrations involved in these studies, becomes reinvolved in exchange. The extent of exchange between glutamine and phosphate was determined at 1000 and 10 μM NH_4^+ during the course of synthesis of 600 nmol of glutamine. The upper limit of the extent of oxygen exchange between glutamine and phosphate was calculated by assuming that 1000 nmol of glutamine and 600 nmol of P_i were present throughout the course of the reaction. As seen in Table III, nearly 3 times more exchange occurred at 10 μM NH_4^+ than at 1000 μM NH_4^+. However, even the calculated upper limit of the amount of exchange which occurred at either concentration was insignificant in comparison to the exchange which occurred between glutamate and phosphate under similar conditions. Therefore, once glutamine is released to the medium it seldom becomes involved in further exchange under the conditions prevailing in these experiments. Nearly all the exchange which occurs between glutamate and P_i occurs prior

to glutamine dissociation. These data then rule out the possibility that the observed NH_4^+ modulation of the $O_{glutamate} \rightleftharpoons O_{P_i}$ exchange is a result of a competition between NH_4^+ and glutamine for a common binding site.

Discussion

The experimental results clearly show that a decrease in the NH_4^+ concentration during glutamine synthesis results in considerable oxygen exchange between glutamate and phosphate. Similar substrate modulations of oxygen exchange occur with *E. coli* succinyl-CoA synthetase (Bild et al., 1980), with soluble mitochondrial ATPase (Choate et al., 1979; Hutton & Boyer, 1979), with membrane-bound ATPase (Russo et al., 1978), and in ATP synthesis by mitochondria and chloroplasts (Wimmer & Rose, 1977; Hackney & Boyer, 1978; Hackney et al., 1979). As has been shown with these systems, the most probable explanation of our results with glutamine synthetase is that the binding of NH_4^+ to a catalytic site of one subunit promotes catalytic events at another site. This could result from the facilitation of the release of one or more products or the decrease in the rate of reaction reversal at another catalytic site.

The success of this approach to evaluate if catalytic cooperativity may occur between subunits of *E. coli* glutamine synthetase is dependent upon the equivalence in reactivity of the oxygens of the bound glutamate γ-carboxyl group and of the bound P_i (Stokes & Boyer, 1976; Balakrishnan et al., 1978). Previous studies (Kowalsky et al., 1956), confirmed here, show that at high substrate concentrations reversal of the oxygen-exchange steps does not occur to any significant extent since only a single glutamate oxygen is transferred to P_i during net glutamine synthesis. Herein lies the power of the present technique, for any reversal of the oxygen-exchange steps before product release is revealed by greater than the required stoichiometric transfer of one oxygen from glutamate to the product P_i.

The observed NH_4^+ modulations are consistent with catalytic cooperativity between sites. Which particular catalytic step or steps are modified by NH_4^+ (or NH_3) binding is not known. Measurements of the relative rates of several exchange reactions at saturating substrate concentration (Wedler, 1974) suggest that ADP is the last product released. NH_4^+ (or NH_3) binding must make release of P_i and glutamine preferable to the formation of ATP and glutamate. Marked slowing of P_i and glutamate release might result in decreased enzyme turnover. It seems quite possible that release of all substrates including ADP at one site could be promoted by NH_4^+ binding at an alternate site.

In contrast to the results obtained with NH_4^+, lowering of ATP concentration resulted in only a small amount of oxygen exchange. A definite modulation by ATP occurs—it is just much less impressive than the NH_4^+ modulation. The small modulation of the $O_{glutamate} \rightleftharpoons O_{P_i}$ exchange by ATP provides an important control since longer incubation times are also used at low ATP concentrations. Thus, exchange by some unknown enzyme is unlikely. Furthermore, to our knowledge, no enzyme other than glutamine synthetase is known that catalyzes oxygen exchange between glutamate and P_i.

Consideration has not been given here to any isotope rate effects. A small isotope rate effect of the ^{18}O could occur during P–O and C–O bond breakage during substrate interconversion. However, any such small effects would not alter our interpretations of the data.

The evidence that the increased exchange occurs by reactions of bound products prior to their release needs to be considered. First, does the exchange involve only bound ADP formed from medium ATP? Although the large excess of pyruvate kinase rapidly converts medium ADP to ATP, a finite level of free ADP must be present in the medium. This concentration of medium ADP would be expected to be lower at low NH_4^+ concentration where the rate of synthesis of ADP is much slower. Therefore, if medium ADP must bind for exchange to be observed, greater exchange per mole of product formed would be observed at high NH_4^+ concentration, contrary to that observed experimentally. More important is the evidence that under the conditions used, medium glutamine participates only slightly in the exchange (Table III). When glutamate and ATP are present, as in the present experiments, they likely bind preferentially to binding of glutamine and P_i, blocking the relatively rapid $O_{gln} \rightleftharpoons O_{P_i}$ exchange that can occur under other conditions (Stokes & Boyer, 1976). Since glutamate has been shown to bind tightly in comparison to the interconversion steps and product release (Stokes & Boyer, 1976), it is unlikely that there is significant oxygen exchange between medium glutamine and medium glutamate.

With respect to participation of bound P_i, present information does not eliminate the possibility that P_i could rapidly dissociate, equilibrate with medium P_i, rebind, and undergo further exchange. However, this would not alter the interpretation that at low NH_4^+ concentration more reversals of bound glutamate formation from bound glutamine occur prior to glutamine release.

Other possible explanations of our results need consideration. The NH_4^+ modulation of the exchange reaction could be the result of a change in catalytic flux through two different reaction pathways, one resulting in no reversals of the exchange reaction and the other leading to numerous reversals. Such a situation, as previously considered for *E. Coli* succinyl-CoA synthetase (Bild et al., 1980), chloroplast ATP synthetase (Hackney et al., 1979), and soluble mitochondrial ATPase (Hutton & Boyer, 1979) could be a consequence of several phenomena. (a) One is enzyme hysteresis, where an enzyme form (E) gives catalysis with much exchange but a different form (E′), present immediately after product release, gives catalysis without exchange. At low NH_4^+ a slow transition of E′ to E occurs prior to substrate binding resulting in catalysis with exchange. However, at high NH_4^+ the transition cannot occur before substrate binds, and catalysis occurs without exchange. (b) Another is enzyme heterogeneity where there are two forms of the enzyme, one with a high K_m for NH_4^+ and the other form with a low K_m. The low K_m form is present as a minor fraction of total enzyme activity. (c) A third is control sites where at high NH_4^+ the control site is occupied and little exchange occurs and at low NH_4^+ the control site is vacant and extensive exchange occurs. Such possible explanations for the observed substrate modulation were eliminated in the case of *E. coli* succinyl-CoA synthetase (Bild et al., 1980) and chloroplast (Hackney et al., 1979) and mitochondrial ATPases (Hutton & Boyer, 1979). Mass spectral analysis of the $^{18}O/^{16}O$ distribution among the five species of inorganic phosphate (i.e., $P^{16}O_4$, $P^{16}O_3{}^{18}O_1$, $P^{16}O_2{}^{18}O_2$, $P^{16}O_1{}^{18}O_3$, and $P^{18}O_4$) showed that all the $[^{18}O]P_i$ species were produced as a result of a single catalytic pathway. This technique was applicable to these enzymes because the analyzed P_i had undergone exchange with a large pool of succinate, in the case of succinyl-CoA synthetase, or water, in the cases of the mitochondrial and chloroplast ATPases. However, with *E. coli* glutamine synthetase, P_i undergoes exchange with bound glutamate, a substrate that does not rapidly equilibrate with the large pool of medium glutamate (Stokes & Boyer, 1976). Because exchange is limited to bound glutamate, the

average maximum number of ^{18}O oxygens derived from glutamate (84% labeled) which could appear in each P_i formed if infinite reversals occur would be 1.34 (the two γ-carboxyl glutamate oxygens would be distributed among four P_i oxygens and one amide oxygen of glutamine after infinite reversals). Thus, there would not be present adequate amounts of P_i containing two to four ^{18}O per P_i to enable differentiation between one pathway or two pathways for formation of P_i. Therefore, enzyme hysteresis, enzyme hetereogeneity, or control sites, although regarded as unlikely, are not conclusively eliminated as possible explanations of the data.

If the basis for the NH_4^+ modulation is catalytic cooperativity, NH_4^+ may be expected to induce important conformational changes in the enzyme. Studies with fluorescence and stopped-flow techniques have shown that a major change in the conformation of *E. coli* glutamine synthetase accompanies NH_4^+ binding (Timmons et al., 1974). When NH_4^+ is added to the Mg^{2+}-dependent enzyme·ATP·glutamate complex, there is a rapid quenching of tryptophan fluorescence to the same level obtained by the addition of ADP, P_i, and glutamine to the enzyme. Meek & Villafranca (1980) have reported that double-reciprocal plots of initial velocity data with varied NH_4^+ concentration are nonlinear at subsaturating glutamate concentrations; this suggests a negatively cooperative interaction between NH_4^+ and enzyme which is dependent on the fixed concentration of glutamate.

Other data also demonstrate interactions between catalytic sites on subunits. Rhee et al. (1980) have reported that the rate of inactivation of purified unadenylylated glutamine synthetase by methionine sulfoximine and ATP slows down progessively from the expected first-order rate, an indication that an inactivated subunit retards reactivity of its neighboring subunits. Wedler (1980) has shown changes in kinetic properties of unmodified subunits upon reaction of part of the subunits with methionine sulfoximine. Shrake et al. (1980) have noted negative cooperativity of methionine sulfoximine binding with both the adenylylated and unadenylylated enzyme as measured by perturbation of tyrosine spectra. An extreme case of negative cooperativity occurs with rat liver glutamine synthetase (Tate et al., 1972). With this enzyme, composed of only eight subunits, a sigmoidal relationship between inhibition and binding of methionine sulfoximine was reported in which essentially complete inactivation occurred with the binding of 4.0–4.2 mol of methionine sulfoximine/mol of enzyme, thus, implying that glutamate may only half-saturate the enzyme.

The lack of a detectable ATP $\rightleftharpoons$ ADP exchange in the absence of NH_4^+ (Wedler & Boyer, 1972) and the inability of kinetic studies to disclose the existence of covalent intermediates may find their explanation in the observations reported here. Such phenomena would result if NH_4^+ is needed to facilitate the dissociation of ADP from the enzyme.

In conclusion, the measurement of oxygen interchange between groups with torsional symmetry, carboxylate and phosphate, has been demonstrated to be an excellent probe of possible subunit interactions during catalysis. The approach may be extended to other oligomeric enzymes which catalyze reactions involving substrates with equivalent oxygen atoms.

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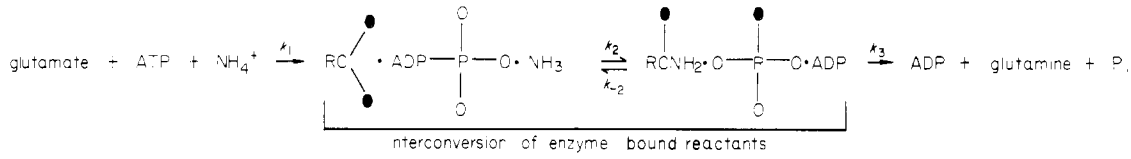
Scheme I



Appendix: Theoretical Relationships for Oxygen Transfer Catalyzed by *Escherichia coli* Glutamine Synthetase during Net Adenosine 5′-Triphosphate Cleavage

For explanation of the approach used Scheme I is useful, in which the oxygens initially present in glutamate are considered to be entirely labeled with ^{18}O, as indicated by a filled O. It is not necessary to consider any catalytic intermediates between bound glutamate and bound glutamine and P_i because oxygen transfer requires reversal of the overall reaction.

The reaction mechanism and stoichiometry require the incorporation of one glutamate oxygen into each P_i formed as shown. On the basis of experimental findings reported or referenced in the accompanying paper (Bild & Boyer, 1980), the release of glutamate once bound and the rebinding of P_i once released are considered to be negligible under the experimental conditions used. A rapid randomization of phosphate oxygens by rotation is considered to result in an equal probability of any one of the four oxygens participating in the reformation of reactants if the reversal of step 2 occurs before product release; such equal participation is in accord with the report of Balakrishnan et al. (1978). The bound carboxylate group of glutamate is also regarded as having rotational freedom, because both its oxygens can participate in exchange with P_i (Stokes & Boyer, 1976). This oxygen equivalence assures the complete equilibration of labeled oxygens between the pool of carbonyl oxygens of glutamine and the oxygens of the P_i pool if a large number of reversals occur. At isotopic equilibrium each P_i molecule may contain one or two ^{18}O atoms, but the average number of ^{18}O atoms present per P_i molecule can not exceed the equilibrium value of 1.6.

The partitioning of bound products between release to the medium and reformation of bound substrates is governed by P, the probability of the reversal of step 2 of reaction Scheme I.[3] The value for $\bar{O}'$, the average number of oxygens from glutamate found per P_i released, is given by eq A1. This

$$\bar{O}' = \sum_{n=0}^{\infty} (\text{av. no. of labeled oxygens in } P_i \text{ after } n \text{ reversals}) \times (\text{probability that } n \text{ reversals will occur before product release}) \quad \text{(A1)}$$

equation serves as a useful starting point from which to derive the dependence of R, the average number of reversals, on $\bar{O}'$.

The probability that n reversals will occur before product release is $(1 - P)P^n$. The average number of labeled oxygens present after n reversals, O_n, can be predicted from the average number present after $n - 1$ reversals using eq A2, where a is

$$O_n = \frac{4 - O_{n-1}}{4}\left(O_{n-1} + \frac{2 - O_{n-1}}{2}\right) + \frac{O_{n-1}}{4}\left[(O_{n-1} - 1) + \frac{3 - O_{n-1}}{2}\right] = ab + cd \quad \text{(A2)}$$

the probability that an unlabeled oxygen in P_i will be involved in exchange during reversal n, c is the probability that a labeled oxygen in P_i will be involved in exchange during reversal n,

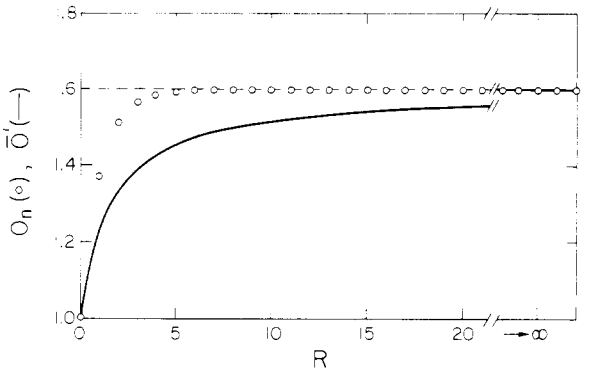


FIGURE A1: Plot of $\bar{O}'$ and O_n vs. R. The curve in this figure shows the dependence of the average number of glutamate oxygens found per phosphate ($\bar{O}'$) on the average number of reversals which occurred during the reaction (R). The theoretical maximum value for $\bar{O}'$ of 1.6 is indicated by the dashed line. The open circles represent the values which would be obtained if one could observe the average number of glutamate oxygens per phosphate in a population of molecules which had all undergone the same number of reversals (O_n). This parameter approaches equilibrium exponentially. It is not represented by a continous curve since O_n is defined only at intergral values of R.

b is the average number of labeled oxygens present in the P_i reformed after reversal n has taken place if reversal n does not involve a labeled oxygen, and d is the average number of labeled oxygens present in the P_i reformed after reversal n has taken place if reversal n does involve a labeled oxygen.

Equation A2 simplifies to

$$O_n = 1 + (3/8)O_{n-1} \quad \text{(A3)}$$

which can be expanded in the following manner:

no. of reversals	O_n
0	$1 = O_0$
1	$1 + (3/8)O_0 = 1 + (3/8) = O_1$
2	$1 + (3/8)O_1 = 1 + (3/8) + (3/8)^2 = O_2$
3	$1 + (3/8)O_2 = 1 + (3/8) + (3/8)^2 + (3/8)^3 = O_3$
.	.
.	.
.	.
n	$\sum_{i=0}^{n}(3/8)^i = O_n$

$$\sum_{i=0}^{n}(3/8)^i = (8/5)[1 - (3/8)^{n+1}] \quad \text{(A4)}$$

Substitutions can now be made into eq A1 to yield eq A5

[3] In the simple scheme shown, the probability of reversal, P, equals $k_{-2}/(k_3 + k_{-2})$ and is identical with the partition coefficient P_c discussed by Boyer et al. (1977) and Hackney & Boyer (1978a). The exchange of oxygens of bound phosphate likely requires reversal of steps to form γ-glutamyl phosphate and cleavage of this intermediate by NH_3 to yield substrates. Thus, the overall rate constants, k_2, k_{-2}, and k_3, of the simplified reaction scheme are complex functions of the rate constants for any participating individual steps.

$$\bar{O}' = \sum_{i=0}^{\infty}(8/5)[1 - (3/8)^{i+1}](1 - P)P^i =$$
$$(8/5)[(1 - P)[1 - (3/8)] + (P - P^2)[1 - (3/8)^2] +$$
$$(P^2 - P^3)[1 - (3/8)^3] + \ldots] =$$
$$(8/5)[[1 - (3/8)] + P[[1 - (3/8)^2] - [1 - (3/8)]] +$$
$$P^2[[1 - (3/8)^3] - [1 - (3/8)^2]] + \ldots] =$$
$$(8/5)\sum_{i=0}^{\infty}[(3/8)^i - (3/8)^{i+1}]P^i \quad \text{(A5)}$$

but

$$(3/8)^i - (3/8)^{i+1} = (3/8)^i[1 - (3/8)] = (5/8)(3/8)^i$$

Therefore

$$\bar{O}' = \sum_{i=0}^{\infty}(3/8)^i P^i$$
$$= \frac{1}{1 - (3/8)P} = \frac{8}{8 - 3P} \quad \text{(A6)}$$

This equation predicts that if the probability of reversal, P, is zero then no exchange will occur and only one glutamate oxygen will be incorporated into phosphate. If the probability of reversal is 1, then infinite exchange will occur and the equilibrium value of 1.6 will be observed for the average number of glutamate oxygens present per P_i molecule released.

The average number of reversals is also related to the probability of reversal by the infinite series [see Hackney and Boyer (1978a)]:

$$R = \sum_{i=1}^{\infty} i(1 - P)P^i = \frac{1}{1 - P} \quad \text{(A7)}$$

Rearrangement and simplification yield

$$P = \frac{R}{R + 1} \quad \text{(A8)}$$

From eq A6 and A8 it follows that

$$R = \frac{8\bar{O}' - 8}{8 - 5\bar{O}'} \quad \text{(A9)}$$

$$\bar{O}' = \frac{8(R + 1)}{5R + 8} \quad \text{(A10)}$$

By use of a similar approach, relations such as those expressed in eq A6, A9, and A10 have been derived for the exchange between water and phosphate oxygen pools which occurs during hydrolysis by enzymes such as the mitochondrial and chloroplast ATPases, sarcoplasmic reticulum ATPase, and yeast pyrophosphatase (Hackney & Boyer, 1978ab; Boyer et al., 1977). Figure A1 shows how the number of glutamate oxygens in the product phosphate ($\bar{O}'$) approaches equilibrium as the number of reversals increases. As is evident from the curve, there is a fairly wide range in which the average number of reversals can be accurately determined from experimentally observed values of $\bar{O}'$. R does not show an exponential approach to the limiting value of 1.6. Such a first-order relation would apply in cases where the exchange occurs at equilibrium between relatively large reactant pools. However, under the conditions used in this study, the exchange of glutamate oxygens is essentially confined to the glutamate initially bound. The observed $\bar{O}'$ thus reflects the average exchange occurring prior to the release of glutamine. That the exchange is in one sense exponential in nature is shown by a hypothetical consideration. Assume that one could measure the average number of glutamate oxygens in a large population of phosphate molecules, each of which had undergone n reversals (O_n, see eq A3). The relationship between these values and R, represented by the open circles, does show a rapid, first-order approach to equilibrium.

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