^{15}N Isotope Effects in Glutamine Hydrolysis Catalyzed by Carbamyl Phosphate Synthetase: Evidence for a Tetrahedral Intermediate in the Mechanism†

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ABSTRACT: ^{15}N isotope effects have been measured on the hydrolysis of glutamine catalyzed by carbamyl phosphate synthetase of *Escherichia coli*. The isotope effect in the amide nitrogen of glutamine is 1.0217 at 37 °C with the wild-type enzyme in the presence of MgATP and HCO_3^- (overall reaction taking place). This *V*/*K* isotope effect indicates that breakdown of the tetrahedral intermediate formed with Cys 269 to release ammonia is the rate-limiting step in the hydrolysis. A full isotope effect of 1.0215 is also seen in the partial reaction catalyzed by an E841K mutant enzyme, whose rate of glutamine hydrolysis is not affected by MgATP and HCO_3^-. With wild-type enzyme in the absence of MgATP and HCO_3^-, however, the ^{15}N isotope effect is reduced to 1.0157. These isotope effects are interpreted in terms of partitioning of the tetrahedral intermediate whose rate of formation is dependent upon a conformation change which closes the active site after glutamine binding and prepares the enzyme for catalysis. An Ordered Uni Bi mechanism for glutamine hydrolysis that is consistent with the isotope effects and with the catalytic properties of the enzyme is proposed.

Carbamyl phosphate synthetase (CPS[1]) catalyzes the formation of carbamyl phosphate, an obligatory precursor of pyrimidine and arginine biosynthesis. Carbamyl phosphate is formed from glutamine, HCO_3^-, and two molecules of ATP in three distinct reactions. Glutamine hydrolysis is catalyzed by the amidotransferase subunit at a site remote from the site of its utilization in the synthetase subunit (*1*). This raises a fundamental question: What is the nature of the mechanism that underlies the coupling of the reactions involved in glutamine hydrolysis and carbamyl phosphate synthesis. An experimental attack on this problem requires a prior knowledge of the catalytic mechanism. Although many amidotransferase-catalyzed reactions have been studied (reviewed in ref *2*), the mechanism of glutamine hydrolysis is not well understood. Labeling studies (*3*) with glutamine transition-state analogues and alkylating agents have pointed to the essentiality of a unique cysteine in the active site. Thus, γ-glutamyl transferase, esterase, and hydrolytic activities suggest that water and hydroxylamine are competing for the same product, and that the reaction probably occurs by way of an acyl−enzyme intermediate. The existence of the intermediate was shown first in CPS by Wellner et al. (*4*) and then in hamster CAD (*5*). In the latter studies, Chaparian and Evans established that glutamine hydrolysis proceeds according to an ordered acyl−enzyme mechanism. The minimal mechanism is illustrated by the three-step sequence below:

$$E + S \overset{K_S}{\rightleftharpoons} ES \xrightarrow{k_2} \text{acyl}-E + P \xrightarrow{k_3} E + Q \qquad (1)$$

where K_S is the substrate dissociation constant k_{-1}/k_1, ES is the Michaelis complex, acyl−E is the covalent enzyme intermediate, P is nascent NH_3, and Q is glutamic acid.

The kinetic properties of three Class I amidotransferases are in accord with the minimal mechanism. Thus, glutamine hydrolysis proceeds through a thiolester intermediate in which glutamine is transferred to a homologous cysteine residue in the reactions catalyzed by hamster CAD (*5*), CPS (*6*), and *p*-aminobenzoate synthase (PabA) (*7*). An acid-stable form of enzyme-bound glutamine has been detected in each of the enzymes. The acyl intermediate formed in CPS has been isolated and chemically characterized (*8*). Its absence in a mutant enzyme in which Cys 269 of the amidotransferase is replaced by a serine provided compelling evidence for the identity of the covalent adduct as the γ-glutamyl group in ester linkage with cysteine (*8*). This has been confirmed by direct visualization of the thiolester in the crystal structure of CPS (*9*) in which the half-life of the compound was increased as a result of a His 353→Asn substitution in the amidotransferase subunit. In the steady state and at saturating concentrations of glutamine, the enzyme-bound intermediate accumulates to average molar ratios of 0.31, 0.56, and 0.89 determined in CPS (*6*, *8*), PabA (*10*), and CAD (*5*), respectively. The kinetics of enzyme deacylation determined directly (*5*) or by steady-state analysis of thiolester formed (*6*, *7*) give ratios of the rate constants k_2 and k_3 (mechanism 1) which quantitatively account for the values of k_{cat} and K_m

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[1] Abbreviations: CPS, carbamyl phosphate synthetase; carbamyl-P, carbamyl phosphate; PEP, phosphoenolpyruvate; APAD, 3-acetylpyridine adenine dinucleotide; CAD, carbamyl phosphate synthetase−aspartate transcarbamylase−dihydroorotase multifunctional complex; chloroketone, L-2-amino-4-oxo-5-chloropentanoic acid.

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measured in the hydrolytic reaction. Roux and Walsh (*7*) note that in the steady state $k_3 = 0.8k_2$, which suggests that breakdown of the thiolester is not totally rate-limiting and k_2 also contributes to k_{cat} in the glutaminase reaction. That the thiolester is a kinetically competent intermediate in overall carbamyl phosphate synthesis has been shown by studies (*11*) in which the observed rates of thiolester formation and hydrolysis, measured in pulse-chase and rapid-quench experiments, were consistent with catalytic turnover determined in the overall reaction under the same conditions.

From mutagenesis studies there is strong evidence for the involvement of a histidine residue in glutamine hydrolysis (*12*). The essential His 170 of anthranilate synthase has been proposed to act as a base catalyst that withdraws a proton from Cys 84 thereby increasing the nucleophilicity of the otherwise protonated sulfhydryl. A nearby and invariant glutamic acid (Glu 172), reminiscent of the charge-relay system of the serine proteinases, has led to the proposal (*13*) that the three residues (Cys-His-Glu) constitute a catalytic triad that promotes hydrolysis by the mechanism operative in papain. This, however, is not supported by evidence indicating that an E170D mutation in PabA has only marginal effects on k_{cat} and K_m and an E170A enzyme retains activity (*10*). In the case of PabA (*10*) and CPS (*14*), His 178 and His 353 in the respective amidotransferase subunits have also been shown to be essential for glutamine hydrolysis. The His353Asn mutant enzyme is able to bind glutamine but is at least 200 times slower in substrate hydrolysis. The crystal structures of two amidotransferases, GMP synthase (*15*) and CPS (*9*), are fully consistent with the participation of either a catalytic triad or an interactive Cys-His ion pair in the catalytic mechanism. Both enzymes have histidines sufficiently near to the reactive cysteine for proton transfer. Even though Glu 355 in the active site of CPS could potentially be part of a catalytic triad, there is no evidence that hydrolysis of the amide bond in glutamine requires transfer of a proton other than that donated by histidine.

The near superposition of GMP synthase catalytic site groups on those of papain and the serine proteinases (*15*), and the recently reported structure of the transition-state analogue γ-glutamylsemialdehyde hydrate bound covalently in the active site of CPS (*16*), imply a tetrahedral intermediate in the mechanism of glutamine amide hydrolysis. This intermediate would be the direct precursor to release of NH_3 and formation of the thiolester. While a tetrahedral intermediate has been mentioned in recent discussions of glutamine hydrolysis (*9*, *14*, *16*), experimental evidence for its existence has not been adduced. Heavy atom isotope effects provide a powerful means of obtaining information about the rate-determining step or any other moderately slow event preceding the first irreversible step of a reaction (*17*, *18*). The reversible formation of a tetrahedral intermediate followed by bond breakage would be expected to be subject to a large isotope effect. In the present study we have measured enrichment of naturally occurring ^{15}N in glutamine as a test for the rate-limiting step in its conversion to glutamate and ammonia.

MATERIALS AND METHODS

Materials. ATP and ADP were obtained from Pharmacia BioTech. Phosphoenolpyruvate (PEP), NADH, 3-acetylpyridine adenine dinucleotide (APAD), glutamine, and cacodylic acid were purchased from Sigma. Type VI pyruvate kinase (rabbit muscle), lactate dehydrogenase (rabbit muscle), and Type II glutamate dehydrogenase (bovine liver) were also purchased from Sigma. Nessler's reagent was obtained from Fluka Chemicals and was used according to instructions provided by the manufacturer. AG 50W-X2 cation-exchange resin (hydrogen-form, 100−200 mesh) was purchased from Biorad. All other chemicals were of the highest purity commercially available.

Enzyme Preparation. Wild-type CPS (specific activity, 4.2 μmol carbamyl-P and glutamate formed at 37 °C min^{-1} mg^{-1}) and the E841K mutant holoenzyme (*19*) were purified respectively from *E. coli* strains L852 and L871 harboring recombinant plasmids (pLLK12 and pLL43) carrying the wild-type or mutant *carAB* operon, as previously described (*19*). The proteins were stored at −80 °C as suspensions in 100 mM potassium phosphate (pH 7.6), containing 5 mM ornithine and 2 M ammonium sulfate. For use in the ^{15}N isotope experiments, the protein was transferred to 50 mM potassium phosphate buffer (pH 7.4) by centrifugation through a column of Sephadex G-50. After desalting, the enzyme solution was free of NH_4^+ as judged by Nessler's assay. It was clarified and sterilized by passage through a 0.2-μm cellulose acetate filter. Protein concentration was determined in 50 mM potassium phosphate buffer (pH 7.4) from the absorbance at 280 nm, using an extinction coefficient of 0.685 cm^{-1} mg^{-1} ml^{-1} (*20*, *21*).

Assay of CPS-Catalyzed Glutamine Hydrolysis. Hydrolysis of glutamine, either in the partial reaction or in overall carbamyl phosphate synthesis, was determined by measuring the amount of ADP or glutamate formed. Measurement was made by endpoint assay, using the coupled pyruvate kinase−lactate dehydrogenase or NAD−glutamate dehydrogenase systems (*22*). The progress of glutamine hydrolysis was determined from reaction mixtures incubated at 37 °C. Aliquots containing 200 μL of the reaction solution were withdrawn at indicated intervals and transferred to 0.1 vol of 1 N HCl to quench the reaction. After incubation at 0 °C for 10 min, the solution was neutralized by addition of 0.1 vol of 1 M Tris base. The ADP concentration was determined at 23.5 °C by adding 25 or 50 μL of the sample to a cuvette containing 1 mL of an assay solution composed of 100 mM Tris/acetate (pH 7.6), 60 mM KCl, 20 mM $MgSO_4$, 1 mM PEP, 0.3 mM NADH, 5 units of pyruvate kinase, and 10 units of lactate dehydrogenase. The amount of ADP in the sample was calculated from the net decrease in absorbance at 340 nm, using 6.22 as the millimolar extinction coefficient of NADH.

The amount of glutamate in the quenched reaction solution was determined from the increase in absorbance at 363 nm upon conversion of glutamate to α-ketoglutarate with the 3-acetylpyridine analogue of NAD and glutamate dehydrogenase (*23*). Assay solutions (1 mL) contained 100 mM Tris/acetate (pH 8.0), 50 mM $KH_2PO_4^{2-}$, 1 mM ADP (to stabilize the enzyme), 0.5 mM acetylpyridine dinucleotide, and glutamate dehydrogenase (15 units). An appropriate aliquot (20−50 μL) of the neutralized sample containing glutamate was added to the assay mixture so that the final concentration of glutamate was between 0.02 and 0.1 mM. The assay solutions were incubated for 30 min at 37 °C. A blank contained all of the same components except glutamate.

Standards containing 0.02−0.1 μmol of glutamate were included in each experiment. The net increase in absorbance at 363 nm of reduced acetylpyridine dinucleotide was determined, and the concentration of glutamate was calculated, using a millimolar extinction coefficient for APADH of 8.33 obtained from a standard curve. The extent of glutamine hydrolysis at the end of the experiment was determined in triplicate from aliquots of the zero- and 50%-conversion reaction mixtures. These values were used to calculate the precise fraction of reaction (*f*) needed for analysis of the isotope effect.

Isotope Effect Nomenclature. The nomenclature used in this work is that of Northrop (*24*) in which the leading superscript denotes the isotope responsible for the effect on the given kinetic (or thermodynamic) parameter. The ^{15}N isotope effect on *V/K* is written as $^{15}(V/K)$. This expression represents the rate of the ^{14}N-containing species under *V/K* conditions relative to the rate of the ^{15}N-containing species $[(V/K)_{N_{14}}/(V/K)_{N_{15}}]$. To designate the specific substrate on which the isotope effect is measured, a subscript within the parentheses is included. Thus $^{15}(V/K_{Gln})$ denotes the effect of isotopic substitution in the C-5 amide group of glutamine on *V/K* for glutamine.

Isolation of Glutamine Amide-N for Isotope Analysis. Initial attempts to resolve mixtures of glutamine, glutamate, and NH_4^+ were complicated, because of the well-known instability of glutamine during AG 50W-X8 chromatography (*25*). A considerable cyclization of glutamine to PCA and ammonia occurred during chromatographic separation, and only 50% of the unreacted glutamine was recovered. Nearly quantitative recovery of glutamine was achieved, when chromatography was performed at 4 °C with a low-cross-link resin (AG 50W-X2) and increased flow rates. No attempt was made to separate glutamine from glutamate, which were coeluted from the column. AG 50W-X2 was thoroughly cleaned before use. The resin (100 g) was washed with 1 N HCl (until the filtrate was colorless) on a sintered glass funnel and then rinsed with distilled water to remove the excess acid. The potassium form of the resin was prepared by washing with 0.5 M KOH until the filtrate was alkaline. The dark brown resin was bleached by treatment with hypobromite solution (300 mL of 0.5 M KOH to which was added 15 mL of Br_2), rinsed with 0.5 M KOH until the filtrate appeared colorless, and then washed with water. The nearly white resin was converted to the hydrogen form with an excess of 0.5 N HCl and rinsed with water until the effluent was neutral. Resin (0.6 mequiv/mL bed volume) prepared in this manner was packed as 1.6- × 23-cm columns. The reaction mixture, at pH 2, was applied to the column at a flow rate of 2 mL/min, and the effluent was monitored at 280 nm. ATP, bicarbonate, and carbamyl phosphate and its breakdown products were eluted in the void volume. Glutamine, glutamate, and NH_4^+ were retained on the column. The column was washed with 250 mL of cold distilled water, until the absorbance at 280 nm returned to the baseline. The column was then eluted at a flow rate of 0.7 mL/min with a freshly prepared solution of 0.5 M NH_4-OH, and 7 mL fractions were collected. Glutamine and glutamate eluted as a sharp peak at the solvent front. Fractions found to contain glutamine and glutamate by the ninhydrin spot test were combined. The solution was adjusted to pH 11.5 with about 1.5 mL of 1 M KOH, and the bulk of NH_3 was removed at 10 °C by rotary evaporation. The resulting syrupy residue was dissolved in 10 mL of distilled water and lyophilized to dryness. This step was repeated until the ammonia content of the solution represented <0.1% of the total glutamine. This amount of ammonia does not interfere with the measurement of the isotope effect. The dried residue was dissolved in about 4 mL of distilled water, neutralized with 1 N HCl, and then brought to a known volume with water. The solution was split into three portions for isotope analysis. Glutamine amide-N was completely hydrolyzed by addition of 2 vol of 3 N HCl and heating at 100 °C for 2 h in an oven (*26*). The hydrolysates, cooled to 4 °C, were brought to pH 2 with 5 M KOH and then adjusted to pH ∼5.5 with 1 M KOH. The ammonia released from glutamine was recovered by alkaline steam distillation (*27*). The distilling flask (containing ∼2 mL of water, 0.6 g of MgO, and 0.7 mL of 1 M KOH) was attached to the distillation apparatus, and the steam supply was vented. The hydrolysate and combined washes were introduced through the entrance reservoir in a volume of 10−15 mL. The distillate (30 mL) was collected in a flask containing 5 mL of 0.1 N H_2SO_4 into which the condenser outlet was immersed. The acidic distillate was concentrated to a volume of 2−2.5 mL by heating at 100 °C. All of the ammonia in the hydrolysate was recovered in the distillate. The overall recovery of ammonia from the unreacted glutamine was 93.8%.

Isotopic Analysis. For isotope ratio analysis, the concentrated distillate was transferred to one arm of a Y-tube and the remaining arm was filled with 2 mL of sodium hypobromite solution (*28*). The solutions were frozen with dry ice−2-propanol and the Y-tube was evacuated on a vacuum line. The solutions were thawed and mixed *in vacuo* to oxidize the ammonium to N_2. The N_2 was distilled on a high-vacuum line through dry ice−2-propanol and liquid nitrogen traps and was collected on molecular sieves cooled with liquid nitrogen. The purified N_2 was analyzed on a Finnegan Delta isotope ratio mass spectrometer to determine the abundance of ^{15}N.

^{15}N Isotope Effects Determined in Overall Carbamyl-P Synthesis. The isotope effect in the glutamine-dependent synthesis of carbamyl phosphate was determined from reaction mixtures where zero- or 50% of the substrate had been converted to products. The reaction mixture (pH 7.40) contained 30 mM sodium cacodylate buffer, 90 mM KCl, 25 mM $MgCl_2$, 10 mM ATP, Na salt, 10 mM $KHCO_3$, and 5 mM natural-abundance glutamine in a final volume of 90 mL. The reaction was initiated by adding the wild-type holoenzyme (1.2 mg) to the mixture at 0 °C. A 30 mL portion of the solution (zero-conversion sample) was transferred to a separate flask, where the reaction was quenched by addition of 0.12 vol of 1 N HCl (pH ∼1.3). The remaining 60 mL of the reaction mixture was incubated at 37 °C with slow stirring until 50% of the initial substrate had been converted to glutamate. The progress of the reaction was monitored by removing aliquots of the reaction mixture for ADP and glutamate determinations. After 150 μmol of glutamate had been formed (∼120 min), the reaction was stopped by addition of 0.12 vol of 1 N HCl (pH 1.35). Under these conditions, carbamyl phosphate hydrolyzes to yield NH_3, HCO_3^-, and inorganic phosphate (*29*). The mixtures were filtered through YM-30 units (Amicon) at 4 °C to remove

denatured protein. At this point, the fraction of reaction was determined by removing an aliquot (100 μL) for glutamate analysis. Analyses were also performed on an aliquot removed from the zero-conversion sample immediately after quenching of the reaction. The filtered solution was diluted with an equal volume of distilled water and adjusted to pH 2. The unreacted glutamine was isolated, and the isotope ratio of the ammonia released from glutamine was determined. In parallel experiments, the ^{15}N to ^{14}N ratios of the starting glutamine and of glutamine isolated from the zero-conversion sample by AG 50W-X2 chromatography were determined by mass spectrometry after hydrolysis of amide-N in 2 N HCl, recovery as ammonia, and oxidation to N_2 with hypobromite as described above.

Determination of ^{15}N Isotope Effect in CPS-Catalyzed Glutamine Hydrolysis. The isotope effect was determined in the glutaminase reaction in the absence of carbamyl phosphate synthesis. Because the slow rate of glutamine hydrolysis in the absence of MgATP and HCO_3^- necessitated longer incubation times, the experiments were done under sterile conditions. The reaction mixture (pH 7.42) contained 20 mM potassium phosphate buffer and 5 mM natural-abundance glutamine in a total volume of 150 mL. The reaction was initiated at 0 °C by adding 12 mg of the wild-type CPS holoenzyme. Part of the reaction mixture (50 mL) corresponding to the zero-conversion sample was quenched by transfer to a flask containing 5 mL of 1 N HCl (pH 1.5). The remaining 100 mL of reaction mixture was incubated at 37 °C with slow shaking. Nonenzymatic hydrolysis of glutamine was measured in parallel by omitting the enzyme. Glutamine hydrolysis was followed by withdrawing 200 μL of the reaction mixtures and measuring the amount of glutamate formed. When 40% of the substrate had been hydrolyzed in the enzymatic reaction (22 h), the reaction solution was acidified, and the denatured protein was removed by filtration through a YM-30 unit at 4 °C. The fraction of reaction was determined from the total glutamate formed. No glutamate or ammonia was detected in the nonenzymatic control. The filtrates obtained from the zero- and 40%-conversion samples were diluted with an equal volume of water, adjusted to pH 2, and applied to 1.6- × 15-cm columns of AG 50W-X2 (H^+-form). Glutamine and glutamate were isolated by chromatography and the amide-N was recovered as N_2 by the same procedures, as described for the overall synthesis.

Analysis of Glutamine Hydrolyzed by Mutant E841K CPS. The isotope effect was also measured in glutamine hydrolysis catalyzed by a mutant holoenzyme. This mutant enzyme catalyzes no carbamyl-P synthesis (*19*) and exhibits altered properties in glutamine hydrolysis (*6*). The isotope experiments with the mutant enzyme were performed according to the same details outlined above for the wild-type enzyme, except that the reaction was performed at pH 6.60 which is close to the pH optimum of the mutant enzyme. The sterile reaction mixture contained 20 mM potassium phosphate buffer and 5 mM natural-abundance glutamine in a volume of 90 mL. The reaction was initiated (at 0 °C) by adding 3 mg of mutant holoenzyme. The zero-conversion sample (30 mL) was treated according to the usual procedure. The remaining 60 mL was incubated at 37 °C for 140 h resulting in 43.4% conversion of the substrate. The reaction was linear over this period, indicating complete stability of the enzyme.

Table 1: ^{15}N Isotope Effects on the Hydrolysis of Glutamine in the Overall and Partial Reactions Catalyzed by Wild-Type and E841K Mutant CPS Holoenzymes

enzyme	reaction catalyzed	pH	f	$^{15}(V/K_{Gln})^a$
wild-type	carbamyl-P synthesis[b]	7.40	0.542	1.0217 ± 0.0002 ($n = 3$)
wild-type	carbamyl-P synthesis[b]	7.40	0.534	1.0217 ± 0.0014 ($n = 3$)
wild-type	glutamine hydrolysis[c]	7.42	0.404	1.0157 ± 0.0001 ($n = 3$)
E841K	glutamine hydrolysis[c]	6.60	0.4337	1.0215 ± 0.0002 ($n = 3$)

[a] The values indicated are the mean and root-mean-square deviation of the measurements made in separate samples of the reaction mixtures. [b] Conditions: 37 °C, 30 mM sodium cacodylate buffer, 10 mM ATP (sodium salt), 25 mM $MgCl_2$, 90 mM KCl, 10 mM $KHCO_3$, and 5 mM natural-abundance glutamine. [c] Conditions: 37 °C, 20 mM potassium phosphate buffer, and 5 mM natural-abundance glutamine.

The isotope ratios were determined by mass spectral analysis following conversion of the amide-N of the remaining glutamine to N_2.

Data Analysis. The ^{15}N isotope effect describing isotope discrimination in the amide group of glutamine is calculated from the following equation:

$$^{15}(V/K_{Gln}) = \log (1 - f)/\log [(1 - f)(R_s/R_0)] \quad (2)$$

where R_s is the $^{15}N/^{14}N$ ratio in the amide-N in the unreacted substrate after fraction of reaction f, and R_0 is the initial $^{15}N/^{14}N$ ratio in the substrate as determined in the zero-conversion control.

RESULTS

^{15}N Isotope Effects Measured in Overall Carbamyl Phosphate Synthesis and in Glutamine Hydrolysis. Glutamine-dependent CPS catalyzes the reaction

$$\text{glutamine} + HCO_3^- + 2\text{MgATP} \rightarrow \text{carbamyl-P} + \text{MgATP} + P_i + \text{glutamate}^- \quad (3)$$

Kinetic studies (*1*) have shown this reaction is irreversible, and dissociation of the products is more rapid than catalysis. V/K for glutamine is about 1×10^5 s^{-1} M^{-1} at 37 °C, measured by formation of ADP or of glutamate. To assess the isotope effect contributed by the amide cleavage reaction, the $^{15}N/^{14}N$ ratio was measured in the glutamine remaining after 50% of the initial substrate had been converted to products. The isotope ratio of ammonia released from the amide group of the remaining glutamine was determined by isotope ratio mass spectral analysis. In two experiments using natural-abundance glutamine, the average $^{15}(V/K_{Gln})$ effect in the glutamine hydrolytic step of overall carbamyl phosphate synthesis was determined to be 1.0217 ± 0.0010 (Table 1). In the absence of MgATP and HCO_3^-, CPS catalyzes the partial reaction

$$\text{glutamine}^- + H_2O \rightarrow NH_3 + \text{glutamate}^- \quad (4)$$

According to mechanism 1, NH_3 is released before glutamate. If it is assumed that NH_3 leaves the enzyme, its protonation in solvent effectively removes it as a reaction participant and glutamine hydrolysis can be considered irreversible. In this

partial reaction (*4*), glutamine hydrolysis, measured as appearance of glutamate, is slow. *V*/*K* for glutamine is 420 s^{-1} M^{-1} (*6*), which is about 200 times lower than the value observed when hydrolysis is coupled to carbamyl phosphate synthesis. K_m for glutamine (0.15 mM) is essentially the same under both conditions. In the partial reaction also, the isotope ratio was determined as above from the ammonia isolated from 60% glutamine remaining at the end of the reaction. The $^{15}(V/K_{Gln})$ in the amide-N was 1.0157 ± 0.0001, a value significantly smaller than the one measured in the overall reaction.

The E841K mutant enzyme has a lysine in place of Glu 841 in the carbamate active site in the synthetase subunit (*19*). This mutation lowers the activity of the carbamate phosphorylation site, measured as transfer of phosphate from carbamyl-P to ADP, to ~3% of the wild-type enzyme. Carbamyl-P synthesis is undetectable (<0.01%). A pH effect on *V* for glutamine hydrolysis is observed. The glutaminase activity of the mutant enzyme displays a single optimum at pH ~ 6.6, in contrast to the wild-type enzyme which exhibits optima at pH 4.2 and pH 9.3 (*6*). Pertinent to the present studies, addition of MgATP and HCO_3^- elicits no increase in glutamine hydrolysis or in the hydrolysis of γ-glutamyl-hydroxamate (*6*). In the absence of MgATP and HCO_3^-, a 60-fold increase in K_m for glutamine is observed, with a 2-fold increase in the rate of glutamine hydrolysis. *V*/*K* for glutamine is 11 s^{-1} M^{-1}. To examine the effect of E841K on amide cleavage only (where the rate constants for hydrolysis of the thiolester would not apply) the reaction of Cys 269 with a chloroketone analogue of glutamine (L-2-amino-4-oxo-5-chloropentanoic acid; ref *30*) has been determined with the wild-type and mutant enzymes (*6*). In this reaction, $k'_{i,app}$ is the observed first-order rate constant for formation of the covalent enzyme−4-oxonorvaline derivative, and K_{Ia} is the observed dissociation constant of the reversible E−chloroketone complex. $k'_{i,app}/K_{Ia}$ of the wild-type enzyme is 7×10^5 s^{-1} M^{-1} compared to 5×10^3 s^{-1} M^{-1} for the mutant enzyme. The 100-fold reduction in catalytic efficiency of the mutant enzyme, however, is not associated with a significant effect on chloroketone binding (K_{Ia} decreases from 3.3 to 1.7 mM in the mutant enzyme). In view of these altered properties, the nitrogen isotope effect was also determined in the partial reaction catalyzed by E841K CPS. The $^{15}(V/K_{Gln})$ obtained was 1.0215 ± 0.0002, which is similar to the value observed with wild-type CPS in the carbamyl phosphate synthesis reaction.

DISCUSSION

The ^{15}N isotope effects measured in hydrolysis of glutamine amide-N are close to the values of 1.023 determined in papain-catalyzed hydrolysis of *N*-benzoylargininamide (*31*) and to the maximum ^{15}N isotope effect of 1.025 obtained in organic model reactions in which C−N bond breaking is known to be rate-determining (*31*, and references therein). In the present studies, the ^{15}N isotope effects are useful in dissecting the mechanism of the CPS-catalyzed glutaminase reaction, because the only step in the hydrolysis that should give a sizable isotope effect is the C−N cleavage that produces ammonia. Assuming this step is irreversible, all subsequent steps have no effect on *V*/*K* for glutamine, and ^{15}N isotope effects determined by the internal competition method are those on *V*/*K*.

A more detailed part of the mechanism than that shown in eq 1 is

$$E + Gln \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{-}Gln \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E{-}Gln^* \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E{-}I \xrightarrow{k_7} [E{-}Glu] + NH_3 \quad (5)$$

where E−Gln is the collision complex of glutamine and enzyme presumably in an open form, E−Gln* is the E−Gln complex after the conformation change which closes the active site and sets the stage for catalysis, E−I is the tetrahedral intermediate, and [E−Glu] is the acyl−enzyme which is subsequently hydrolyzed. In mechanism 5, the *V*/*K* for glutamine is given by

$$V/K_{Gln} = k_1k_3k_5k_7E_t/(k_2k_4k_6 + k_2k_4k_7 + k_2k_5k_7 + k_3k_5k_7) \quad (6)$$

and the ^{15}N isotope effect, assuming that only k_7 will be isotope-sensitive,[2] is

$$^{15}(V/K_{Gln}) = (^{15}k_7 + c_f)/(1 + c_f) \quad (7)$$

where

$$c_f = (k_7/k_6)(1 + (k_5/k_4)(1 + k_3/k_2)) \quad (8)$$

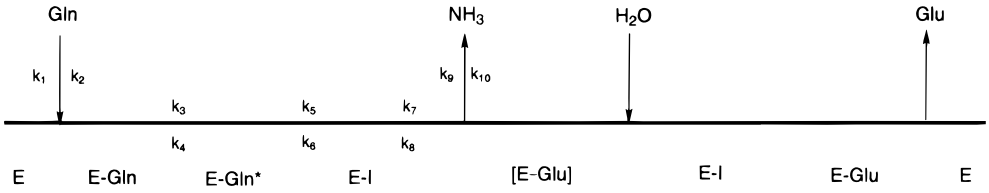
The observation of an ^{15}N isotope effect greater than 2% in the present work shows that c_f is small, and thus none of the partition ratios in eq 8 is very large.[3] In particular, k_7 must be smaller than k_6, which means breakdown of the tetrahedral intermediate to release NH_3 is the major rate-limiting step. This is reasonable, because a proton must be put on the NH_2 group (presumably from His 353 (*16*)) to permit its departure, whereas reversal of tetrahedral intermediate formation requires only departure of the thiolate of Cys 269, and no proton shifts are involved. The observed ^{15}N isotope effect should be pH-independent, because c_f is small.

It is interesting that the ^{15}N isotope effect is the same for wild-type enzyme catalyzing the overall reaction and for the E841K enzyme which is catalyzing only the glutaminase reaction. However, the wild-type enzyme in the absence of MgATP and HCO_3^- gives a reduced ^{15}N isotope effect of 1.57%. This suggests that one of the partition ratios in eq 8 has become larger, so c_f is now ~0.4 (if it was <0.1 to start with). The ratio k_3/k_2 is unlikely to have increased, because the reaction in the absence of MgATP and HCO_3^- is slower than that in its presence and it is unrealistic to think that glutamine would become partly sticky. The ratio k_7/k_6 is also unlikely to increase, because this would require k_6 to decrease

[2] Any ^{15}N isotope effect on the formation of the tetrahedral intermediate will be a secondary one and much smaller than the primary isotope effect on k_7 observed in the present studies. In the case of the aspartate transcarbamylase reaction, the value of the isotope effect on formation of the tetrahedral adduct is 1.0025 (*32*).

[3] Release of NH_3 requires protonation of the $-NH_2$ group. This can take place either at the tetrahedral intermediate or concerted with C−N bond cleavage. In the former case, the observed ^{15}N isotope effect is the product of an inverse isotope effect for formation of the zwitterionic tetrahedral intermediate multiplied by the normal one for its breakdown. In the latter case, one is simply comparing the transition-state structure with that of free glutamine. In either case, the observation of a 2% ^{15}N isotope effect shows clearly that one is looking at a primary isotope effect for C−N cleavage.

Scheme 1



relative to k_7 (for a slower reaction, k_7 certainly would not increase). Thus, the likely culprit is k_5/k_4. This ratio would increase if k_4 decreased. We suspect that in the absence of MgATP and HCO_3^- the conformation change that converts E−Gln to E−Gln* is slow, and that both k_3 and k_4 will be decreased. Possibly k_3 is decreased more than k_4 (the ratio of rates with and without MgATP and HCO_3^- is 200 (*4*, *6*)), so that k_3 becomes the slow step in the reaction. The value of c_f in eq 8 is only dependent, however, on the partition ratios and not on the absolute values of the rate constants. But V/K_{Gln}, as given by eq 6, does depend on the absolute value of k_3, and a low value of k_3 would decrease V/K and the turnover number.

Compared to the ^{15}N isotope effect in glutamine hydrolysis catalyzed by the wild-type, the mutant enzyme shows an increased isotope effect of 2.15% in the glutamine reaction. This suggests that c_f is low (<0.1) in this reaction and one or more of the partition ratios has become smaller as a result of the mutation. k_3/k_2 will have no significant effect on commitment (eq 8), because k_3 is the slow step in the reaction and $k_2 \gg k_3$. It is the ratio k_5/k_4 which represents the most sensitive step in the reaction, and therefore k_5/k_4 must decrease. The isotope effect of greater that 2% in the mutant-catalyzed reaction indicates that the values of k_5/k_4 and k_7/k_6 are similar to or less than those of the wild-type measured in the presence of MgATP and HCO_3^-. The high K_m for glutamine seen with the mutant implies an increase in k_4 and k_2, and, thus, the conformation change converting E−Gln to E−Gln* and possibly the initial binding of glutamine are disfavored. The low turnover number of the mutant (0.05 s^{-1} and 0.1 s^{-1}, compared to the value of the wild-type, 11.2 s^{-1} and 0.063 s^{-1}, with and without MgATP and HCO_3^-, respectively) is presumably limited by k_3.

The observed ^{15}N isotope effects approaching the maximum discrimination ratio are interpreted as clear evidence that C-5 amide bond breaking with release of NH_3 is completely rate-determining in the first step of carbamyl-P synthesis. The full isotope effect can be obtained only if the reaction proceeds by way of the reversible formation of a tetrahedral intermediate followed by its irreversible breakdown. The isotope effects also provide the first experimental proof that NH_3 released by amide-bond cleavage is the chemically reactive species in glutamine amide-N transfer reactions catalyzed by the enzymes of Class I. Previously, this has been inferred on the strength of chemical arguments alone (*30*, *33*).

An important feature of the mechanism we put forward is a conformation change which closes the active site and positions the substrate correctly for the ensuing reaction. Also important for effective catalysis may be subtle changes in the positions of Cys 269 and His 353 favorable for proton transfer. We suspect the conformation change which converts E−Gln to E−Gln* is functionally identical to the well-known conformation change which occurs upon binding of glutamine (*4*, *21*) or upon reaction of cysteine 269 with chloroketone (*34*) or cyanate (*35*) analogues of glutamine. Evidence that this conformation change is engaged in catalytically important and reciprocating interactions with the carboxyphosphate site in the synthetase subunit can be found in refs *4*, *21*, *22*, and *34*−*36*.

In summary, the ^{15}N isotope effects observed in carbamyl phosphate synthesis and glutamine hydrolysis catalyzed by wild-type CPS and a Glu 841→Lys mutant form are consistent with the mechanism of glutamine−amide cleavage described in eq 5. Even though it is no longer sensitive to ^{15}N, the second part of the glutamine hydrolytic reaction is a reverse of glutamine cleavage. The acyl intermediate [E−Glu] is hydrolyzed in an attack by H_2O to give a tetrahedral intermediate, E−I, which breaks down to glutamate and the free enzyme. Glutamine hydrolysis can be described by a Ping Pong Bi Bi mechanism with H_2O as the second substrate and isomerization of the enzyme−substrate and the [E−Glu] complex as diagrammed in Scheme 1 or formally as an Ordered Uni Bi mechanism (*37*).

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