Substitution of Glu841 by Lysine in the Carbamate Domain of Carbamyl Phosphate Synthetase Alters the Catalytic Properties of the Glutaminase Subunit†

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Received April 17, 1992; Revised Manuscript Received November 20, 1992

ABSTRACT: In previous studies a Glu841 → Lys replacement in the carbamate phosphorylating domain located in the COOH half of the synthetase subunit of *Escherichia coli* carbamyl phosphate synthetase was shown to reduce overall synthesis of carbamyl phosphate by 4 orders of magnitude with either glutamine or NH_3 as nitrogen donor (Guillou et al., 1992). In the present study, the mutant enzyme has been further analyzed for its glutamine hydrolytic activity. The glutaminase activity of the mutant enzyme has the following properties. (1) In the absence of other substrates the turnover number is only marginally different from that of the wild-type complex. (2) The K_m for glutamine is 60 times higher than in wild-type complex and three times higher than in the separated glutaminase subunit. (3) In the present study wild-type carbamyl phosphate synthetase has been shown to catalyze glutamine hydrolysis by a mechanism involving an enzyme-bound acyl ester intermediate (γ-glutamyl thioester). This intermediate is formed and is hydrolyzed with rates consistent with overall glutamine hydrolysis. At physiological concentrations of glutamine (1.2 mM), the steady-state concentration of γ-glutamyl thioester is 0.3 mol/mol of wild-type enzyme. Under the same conditions, only 0.02 mol of thioester is measured in the mutant enzyme. Maximal accumulation of this covalent intermediate by the mutant enzyme required 10 times higher concentrations of free glutamine. (4) The rate of reaction with 2-amino-4-oxo-5-chloropentanoate, a glutamine analog known to specifically alkylate an active site cysteine residue, is 2 orders of magnitude slower in the mutant. (5) Binding of both MgATP and bicarbonate to carbamyl phosphate synthetase normally stimulates glutamine hydrolysis by 100-fold. This activation, presumed to be dependent on a carboxyphosphate-induced conformational change of the glutaminase active site, is not observed with the Lys841 enzyme. (6) Finally, the pH dependence of the glutaminase activity in the mutant complex is identical to that of the separated glutaminase subunit which exhibits fewer titratable groups than wild-type holoenzyme. Most of the properties listed above are also displayed by the isolated glutaminase subunit. In addition to the previously reported effects on catalytic activity of the synthetase component, the Lys841 substitution therefore appears to uncouple functional interactions between the glutaminase and carbamate phosphorylation domains.

The synthesis of carbamyl phosphate catalyzed by glutamine-dependent carbamyl phosphate synthetase of *Escherichia coli* is known to depend on interactions between catalytic domains located in the glutaminase and synthetase subunits (Meister, 1989). The synthetase subunit contains two catalytic nucleotide binding domains, one involved in the activation of HCO_3^-, and the second in phosphorylation of carbamate (Meister, 1989; Post et al., 1990; Raushel & Villafranca, 1980). The glutaminase subunit supplies the synthetase with ammonia released from the hydrolysis of glutamine (Trotta et al., 1971).

Even though the glutaminase and synthetase components are capable of expressing their respective enzymatic activities independent of one another, it was manifest from earlier biochemical studies that the two subunits exert significant influence on each other's activity. Thus, binding of glutamine analogs (Pinkus & Meister, 1972) or of cyanate (Anderson & Carlson, 1975) to the glutaminase active site enhances ATPase activity of the synthetase. Functional coupling of the two subunits is also supported by data indicating modulation of glutaminase activity by specific reactions catalyzed on the synthetase. For example, conditions favoring the synthesis of carboxyphosphate from ATP and HCO_3^- stimulate hydrolysis of the glutamine analog, γ-glutamyl hydroxamate, by the glutaminase (Anderson & Meister, 1966). Such observations imply reciprocally induced conformational changes between active sites located on the two different subunits, possibly through one or more shared residues.

As part of a study intended to identify catalytically important amino acid residues in carbamyl phosphate synthetase, we have undertaken a mutational analysis of the *E. coli* enzyme. We have reported a mutation (carB2117) resulting in a Glu841 → Lys841 substitution in a putative ATP binding site of the carbamate phosphorylating domain of the synthetase (Guillou et al., 1992). The mutation reduces carbamyl phosphate synthesis with either glutamine or ammonia by 4 orders of magnitude, but only moderately decreases adenine nucleotide binding by the synthetase, and does not alter the in vivo or in vitro stability of the enzyme or the physical interactions between the glutaminase and synthetase subunits. The alterations in the catalytic properties of the Lys841 enzyme were therefore unlikely to be due to some generalized effect of the mutation on the tertiary structure of the synthetase or on the subunit contact domains. Rather, the data were most consistent with the notion that Glu841 is an important residue in a catalytic region of the synthetase. This conclusion prompted us to examine if and how the Lys841 substitution might affect the catalytic activity of the glutaminase component. On the basis of a comparison of the kinetic and glutamine binding properties of the glutaminase in the wild-type and mutant complexes, we propose that Glu841 is an essential residue for maintaining the active sites of the two subunits in a functionally coupled state.

† These studies were supported by Research Grant GM25846 awarded by the National Institute of General Medical Science.

0006-2960/93/0432-1278$04.00/0

MATERIALS AND METHODS

Reagents. L-[U-^{14}C]Glutamine (270 mCi/mmol) and L-[G-^{3}H]glutamine (56 Ci/mmol) were purchased from Amersham. ATP and NAD were obtained from Pharmacia. L-2-Amino-4-oxo-5-chloropentanoic acid was a gift from Paul M. Anderson (University of Minnesota, Duluth, MN). Other reagents were of the highest quality available commercially.

Purification of Carbamyl Phosphate Synthetases. Wild-type carbamyl phosphate synthetase (3.6 μmol of carbamyl phosphate synthesized min^{-1} mg^{-1} at 37 °C) and Lys841 carbamyl phosphate synthetase were purified respectively from *E. coli* strains L829 and L705, as described previously (Guillou et al., 1992). Both enzymes were estimated to be >98% pure, as judged by Coomassie staining of the proteins after separation on SDS–polyacrylamide gels (Laemmli, 1970). The purified enzymes were desalted by exhaustive dialysis against 0.12 M potassium phosphate, pH 7.5, containing 1 mM EDTA.[1] Solutions of the dialyzed enzymes were clarified by passage through cellulose acetate (0.2-μm) filter units (Nalgene).

Purification of Glutaminase Subunit. Wild-type small subunit was purified to ~85% homogeneity from *E. coli* C600-(Δ*carB8*) harboring a plasmid (pMAC-13/A) that expresses only the *carA* gene. Cells grown in LB medium containing ampicillin (50 μg/mL) were harvested in stationary phase and washed in one-fourth the starting volume of 20 mM Tris/HCl, 1 mM EDTA, pH 7.4. Approximately 10 g wet weight of cells was suspended in 40 mL of 0.1 M potassium phosphate, pH 7.5, containing 1 mM EDTA and 5 mM glutamine. After addition of phenylmethane sulfonyl fluoride to a final concentration of 0.4 mM, cells were disrupted by passage through a French pressure cell (20 000 psi). To the soluble fraction containing the small subunit was added phenylmethane sulfonyl fluoride (0.4 mM) and 0.5 vol of 1.5% protamine sulfate. The precipitate was removed by centrifugation at 20000*g* for 10 min, and the supernatant solution was fractionated with ammonium sulfate. The protein fraction precipitating between 0.35 and 0.65 of saturation was suspended in 5 mL of 0.1 M potassium phosphate, pH 7.5, containing 1 mM EDTA and 5 mM glutamine. The protein (about 200 mg) was applied to a column (3 × 94 cm) of Ultragel AcA34 and eluted with the same buffer containing 0.06% sodium azide. The fractions containing the glutaminase were combined, and the enzyme was precipitated with ammonium sulfate (39.8 g/100 mL). The precipitate was collected by centrifugation and suspended in 5 mL of 0.1 M potassium phosphate, pH 7.5, 1 mM EDTA, and stored at −80 °C. The protein was further purified by chromatography on MonoQ (Pharmacia). The enzyme (100 mg) was desalted by dialysis against 0.05 M potassium phosphate, pH 7.5, 1 mM EDTA, and applied to a column of MonoQ (HR 10/10) equilibrated with the same buffer. The column was eluted with a gradient (0–0.3 M) of NaCl in 0.05 M potassium phosphate, pH 7.5. The fractions containing the peak of glutaminase were combined and precipitated with ammonium sulfate (39.8 g/100 mL), and the precipitate suspended in 3 mL of 0.1 M potassium phosphate, pH 7.1, 1 mM EDTA, was stored at −80 °C.

Protein Determination. The protein concentration of purified carbamyl phosphate synthetase was determined from the absorbance at 280 nm, using a coefficient of A_{280} = 0.685 mg^{-1} (Anderson, 1977; Rubino et al., 1986). The same extinction coefficient was used to estimate the concentration of the glutaminase. This value was considered to be reliable in view of the nearly identical absorbance coefficients of the holoenzyme and of the glutaminase on the basis of their molar contents of tryptophan plus tyrosine. The molar concentrations of the holoenzyme and of the isolated glutaminase were calculated by using molecular weights of 159 100 and 41 400, respectively (Nyunoya & Lusty, 1983; Piette et al., 1984).

Enzyme Assays. Glutaminase activity was determined by measuring the production of glutamate in a stepwise assay with NAD and glutamate dehydrogenase. Reaction mixtures (0.2 mL) contained 50 mM potassium phosphate, pH 7.6, 30 mM glutamine, and 10 or 20 μg of wild-type or mutant protein, respectively. Under these conditions, at the lowest concentrations of glutamine about 15% of the initial substrate was utilized in the reaction. The assays were done in duplicate with and without enzyme at 37 °C. After 60 min the reaction was terminated by addition of 20 μL of 1 N HCl and further incubated at 0 °C for 5 min. Following neutralization with 25 μL of 1 M Tris, the glutamate formed was determined from the fluorescence of NADH after addition of NAD and glutamate dehydrogenase (Brent & Bergmeyer, 1974). The nonenzymatic hydrolysis of glutamine was corrected by subtraction of the glutamate measured in the control samples. K_m and V_{max} (±SD) were calculated by nonlinear least-squares fit of the data to the Michaelis–Menten equation with the program Enzfitter (Leatherbarrow, 1987).

γ-Glutamyl hydroxamate hydrolysis was determined at pH 7.0, at 37 °C, by measuring the disappearance of γ-glutamyl hydroxamate (Anderson & Meister, 1966). Reaction mixtures (0.20 mL) contained 50 mM Hepes/NaOH, pH 7.0, 100 mM KCl, and 5 mM γ-glutamyl hydroxamate. A control containing all of the components of the assay mixture except enzyme was included for each assay. The reaction was started by addition of 25–50 μg of wild-type or mutant enzyme. After incubation for 30–60 min at 37 °C, the reaction was stopped with 0.8 mL of $FeCl_3$ reagent (10% $FeCl_3 \cdot 6H_2O$ dissolved in 0.7 N HCl containing 3% trichloroacetic acid). After a brief centrifugation, the absorbance at 535 nm of the supernatant solution was determined. The amount of γ-glutamyl hydroxamate was estimated from a standard curve.

Assay of Protein-Bound Thioester Intermediate. Formation of the enzyme-bound γ-glutamyl thioester intermediate was determined by measuring the incorporation of [^{3}H]-glutamine into protein-bound glutamate by a modification (Lusty, 1992) of the filter binding procedure described by Chaparian and Evans (1991).

Glutamate Binding. Binding of L-[U-^{14}C]glutamate by wild-type and Lys841 carbamyl phosphate synthetases and the separate wild-type glutaminase subunit was determined under nonequilibrium conditions as described by Wellner et al. (1973), by using the Sephadex centrifugation technique (Penefsky, 1977). The specific conditions used in the binding experiments are described in the appropriate table legend.

RESULTS AND DISCUSSION

Hydrolysis of Glutamine by Free and Complexed Wild-Type and Mutant Glutaminase Subunits. In earlier studies, the isolated glutaminase component of *E. coli* carbamyl phosphate synthetase was shown to hydrolyze glutamate at a rate comparable to that of the native complex. Addition of MgATP and HCO_3^- causes the rate of glutamine hydrolysis by the complex to increase by nearly 2 orders of magnitude (Trotta et al., 1974; Wellner et al., 1973). No enhancement of the hydrolytic rate occurs when these conditions are imposed on the purified glutaminase. These observations have been

[1] Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

Table I: Kinetic Parameters for Glutamine Hydrolysis[a]

carbamyl phosphate synthetase	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
wild type			
holoenzyme	0.15 ± 0.02	0.063 ± 0.016	420
small subunit	2.9 ± 0.4	0.067 ± 0.008	23
Lys841 holoenzyme	9.2 ± 0.7	0.102 ± 0.012	11

[a] Reaction conditions: 37 °C, pH 7.6, 50 mM potassium phosphate, varying concentrations (0.02–50 mM) of glutamine. The values of K_m and k_{cat} are the mean and root square sum of the standard error of several determinations with different preparations of the enzyme.

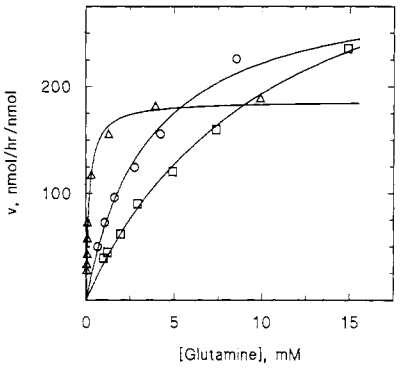

FIGURE 1: Effect of glutamine concentration on the glutaminase activity of wild-type and mutant carbamyl phosphate synthetase and purified wild-type glutaminase. Glutaminase activity was determined at 37 °C in reaction mixtures (0.2 mL) containing 50 mM potassium phosphate, pH 7.6, varying concentrations (0.02–50 mM) of glutamine, and 10–20 μg of wild-type (△), or 25 μg of the Lys841 (□), or glutaminase (○) protein.

confirmed in the present study with the glutaminase subunit purified from a strain of *E. coli* expressing only the *carA* gene. Wild-type carbamyl phosphate synthetase and the purified glutaminase component catalyze hydrolysis of glutamine with maximal rates of 0.063 s^{-1} and 0.067 s^{-1}, respectively (Table I). Addition of MgATP and HCO_3^- results in the expected stimulation of the reaction catalyzed by the complex, but not by the isolated subunit (cf. Table III, below).

A second important difference in the properties of the glutaminase when separated from the holoenzyme is a 20-fold increase in the K_m for glutamine (Figure 1 and Table I). The glutaminase subunit isolated from the *E. coli carA* overproducing strain has a K_m of 2.9 mM[2] for glutamine as compared to the value of 0.15 mM measured with the holoenzyme (Table I). The increased K_m, without any appreciable change of k_{cat}, is also evident in a comparable 18-fold decrease in k_{cat}/K_m, the apparent second-order rate constant for overall acylation of the enzyme.[3] The difference in k_{cat}/K_m (k_3/K_S) is most likely the result of an increase in the dissociation constant (K_S) for glutamine when the glutaminase is dissociated from the synthetase subunit. This conclusion is supported by studies of glutamine binding to the purified subunit and to the holoenzyme.

Mutant carbamyl phosphate synthetase with a Glu841Lys substitution in the synthetase component has properties similar to, but distinct from, those of the isolated glutaminase subunit (Table I). The K_m for glutamine in the mutant complex is 9.2 mM, a value 60 times higher than that measured in wild-type carbamyl phosphate synthetase and three times higher

[2] This value is considerably lower than the K_m of 150 mM reported previously with preparations of glutaminase obtained by dissociation of the holoenzyme with potassium thiocyanate (Trotta et al., 1974). We attribute this discrepancy to the more native state of the enzyme used in the present study.

Table II: Glutamine Binding by Wild-Type and Lys841 Carbamyl Phosphate Synthetases[a]

	[^{14}C]glutamine (plus [^{14}C]glutamate) bound			
	[^{14}C]Gln, 0.12 mM (54 000 cpm/nmol)		[^{14}C]Gln, 1.2 mM (26 250 cpm/nmol)	
enzyme	cpm/50 μL	mol/mol	cpm/50 μL	mol/mol
wt holoenzyme	31989	0.54	22736	0.96
Lys841 holoenzyme	218	0.004	325	0.015
wt glutaminase subunit	nd[b]		16	0.001
wt synthetase subunit	21	<0.001	18	0.001
bovine serum albumin	3		0	
minus enzyme	0		0	

[a] The reaction mixtures contained, in a total volume of 100 μL, 0.14 M potassium phosphate, pH 6.8, 4.8 mM EDTA, and the following amounts of enzyme: wild-type (wt) holoenzyme, 2.35 nmol; mutant holoenzyme, 2.16 nmol; wt small subunit; 1.84 nmol; wt large subunit, 2.37 nmol; bovine serum albumin, 5.65 nmol. The binding reaction was initiated by adding [^{14}C]glutamine to a final concentration of 0.12 mM or 1.2 mM. After incubation for 1 min at 23 °C, free [^{14}C]glutamine was separated from enzyme-bound ligand by centrifugation through a column of Sephadex G-50. The amount of [^{14}C]glutamine (plus glutamate) bound to the enzyme was determined by measuring the radioactivity and the protein concentration of aliquots of the column effluent. [b] nd: not determined.

than in the purified glutaminase. The turnover number of the Lys841 enzyme is 0.102 s^{-1}. This 1.6-fold increase in k_{cat} cannot be interpreted in any straightforward way because of the differences in the pH dependence of the mutant and wild-type enzymes (see Figure 4 below). The increase in k_{cat} of the mutant holoenzyme compared to the isolated glutaminase, however, is meaningful since the two preparations have nearly identical pH profiles. It is also significant that the apparent second-order rate constant k_{cat}/K_m is some two times lower in the mutant than in the small subunit.

Glutamine Binding by Wild-Type and Mutant Carbamyl Phosphate Synthetases. Earlier studies (Wellner et al., 1973) on binding of glutamine to *E. coli* carbamyl phosphate synthetase indicate that the enzyme forms a stable enzyme complex with glutamine. Because of its hydrolysis, a true dissociation constant of glutamine for the enzyme cannot be determined. As discussed in the next section, there is good evidence to suggest that stably bound glutamine consists of glutamine in the form of the enzyme substrate complex and of covalently bound glutamate in the form of a γ-glutamyl thioester.

These properties of carbamyl phosphate synthetase made it of interest to examine whether binding of glutamine to the glutaminase subunit is affected by the Lys841 substitution in the synthetase component. The 60-fold greater K_m of the mutant enzyme suggests that its affinity for glutamine is decreased possibly due to a more rapid k_{off} (hydrolysis and release of glutamate or dissociation of glutamine). This was supported by binding assays using the Sephadex centrifugation procedure to measure [^{14}C]glutamine incorporation. The wild-type and mutant enzymes were incubated for 1 min with a 5- (0.12 mM) or 50-fold (1.2 mM) molar excess of labeled glutamine in 0.14 M potassium phosphate, pH 6.8. At the higher substrate concentration the wild-type holoenzyme bound 0.96 equiv of glutamine/mol of enzyme. Analysis of the radioactivity recovered by quenching the enzyme-bound products with acid revealed approximately equal distribution in glutamine, glutamate, and the thioester intermediate (see following section). The maximum bound by the mutant enzyme was 0.015 mol equiv corresponding to 60 times less than in the wild-type enzyme (Table II). All of this material was determined to be acid-stable thioester. Even though

binding is greatly reduced in the mutant enzyme, it is still significantly greater than in the native glutaminase.

The low affinity of the mutant protein for glutamine precluded a complete titration in the binding studies. Nonetheless, the data summarized in Table II clearly indicate that the Lys841 substitution exerts a remarkable change in the substrate binding properties of the glutaminase component.

Effect of the Lys841 Substitution on the Kinetics of Thioester Intermediate Formation. Approximately 30% of the glutamine bound under the conditions reported in Table II remains complexed to the holoenzyme after acid denaturation. Both the pH stability of the complex and the ability of a chloroketone analog of glutamine to compete in its formation (Pinkus & Meister, 1972) supported the view that the acid stable compound is a γ-glutamyl thioester of the enzyme (Wellner et al., 1973). The identity of the acid stable form of glutamine as a γ-glutamyl acyl ester has been confirmed by the recent demonstration that 0.2–0.4 mol equiv of the enzyme-bound acid resistant form of glutamine is quantitatively converted to γ-glutamyl hydroxamate and its cyclically more stable product pyrrolidonecarboxylic acid by treatment of the complex with hydroxylamine (Lusty, 1992). The fact that a mutant enzyme with a substitution Cys269 → Ser in the active site cysteine residue of the glutaminase component failed to accumulate the acid stable compound further argues for its identity as a γ-glutamyl thioester of cysteine269 (Lusty, 1992).

The effect of glutamine concentration on the steady-state levels of the thioester intermediate in the wild-type and the mutant enzymes is illustrated in Figure 2. In this experiment the maximum amount of intermediate accumulated by wild-type carbamyl phosphate synthetase was 0.36 ± 0.02 mol/mol of enzyme with an apparent K_m for glutamine of 83 ± 14 μM (where $K_{m,app} = K_m\ (k_5/(k_3 + k_5)$ (Gutfreund & Sturtevant, 1956).

The steady-state concentration of 0.25–0.4 mol equiv of thioester measured in the wild-type carbamyl phosphate synthetase suggests that the overall rate of intermediate hydrolysis is only marginally slower than the rate of its formation. With a steady-state concentration of the intermediate of 0.36 equiv, the value of k_3 is about 1.5 times greater than k_5 in the mechanism shown below[3]

Reaction 1

$$E{\cdot}SH + Gln \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{-}SH{\cdot}Gln \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E{-}S{-}Glu + NH_3 \xrightarrow{k_5} E{-}SH + Glu \quad (1)$$

where E–SH represents the active site cysteine of the enzyme, E–SH·Gln is the Michaelis complex, and E–S–Glu, the γ-glutamyl thioester intermediate.

The values obtained for thioester formed as a function of glutamine concentration were used to estimate the kinetic constants of the reaction by least-squares fit to eq 7 (Fersht, 1985) given in footnote 4. The K_m for glutamine obtained from this analysis (assuming $k_2 \gg k_3$) was 110 μM, in good agreement with the value of 0.15 ± 0.02 mM measured by kinetic means (Table I). The analysis also yielded values for k_3 and k_5 of 0.15 s^{-1} and 0.10 s^{-1}, respectively. The participation of the γ-glutamyl thioester as an obligatory intermediate in glutamine hydrolysis requires that the intermediate is both formed and hydrolyzed with rates that are (1) greater than k_{cat} and (2) consistent with the rate of overall

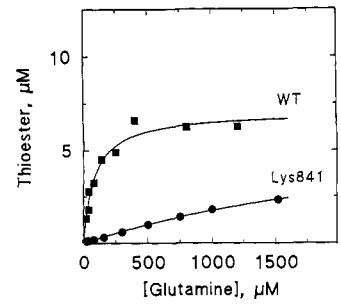

FIGURE 2: Steady-state concentration of thioester as a function of glutamine concentration. Reaction mixtures contained, in a final volume of 50 μL, 0.94 nmol of wild-type (■) or mutant (●) enzyme in 0.05 M Hepes/NaOH, 100 mM KCl, 10 mM potassium phosphate, and 0.2 mM EDTA, at pH 6.9, and [^{3}H]glutamine (21 000 cpm/nmol) at the concentrations indicated in the figure. After equilibration for 2.5 min at 24–25 °C, the reaction was quenched by addition of 1 mL of 5% trichloroacetic acid and 0.1 mL of 0.1 M glutamine. The protein was collected on glass fiber filter discs and washed with HCl, and the amount of [^{3}H]glutamate bound to the enzyme was determined as described in the Materials and Methods section. The curve is a least-squares fit to eq 7, described in footnote 4.

glutamine hydrolysis. The latter is confirmed by substitution of the calculated values of k_3 and k_5 in the expression of k_{cat} (eq 4). Furthermore, the experimentally determined value of k_{cat} of 0.063 is significantly less than the value of k_5 obtained from eq 7 indicating that overall glutamine hydrolysis is affected by the rate of formation as well as by the breakdown of the thioester intermediate.

A similar analysis of the amount of thioester formed with the mutant enzyme gave values of K_m of about 6 mM and 0.15 s^{-1} and 0.1–0.2 s^{-1} for k_3 and k_5, respectively. Although these values are less reliable than those of the wild type, because the titration with glutamine was not extended to saturating concentrations of substrate, the data obtained are consistent with values of k_{cat} and K_m of 0.1 s^{-1} and 9 mM derived from the kinetic data. The mutation does not appear to have any marked effect on the relative rates of formation vs breakdown of the intermediate.

Thioester accumulation is not observed with the isolated glutaminase subunit. The steady-state concentration of the intermediate is 0.016 mol/mol of enzyme, approximately 20

[3] The mechanism of glutamine hydrolysis proposed for all G-type glutamine amidotransferases (Amuro et al., 1985; Chaparian & Evans, 1991; Miran et al., 1991) is based on the acylenzyme mechanism (Gutfreund & Sturtevant, 1956). In the mechanism proposed in eq 1, if formation of the thioester intermediate is accompanied by release of NH_3 from the enzyme, then under conditions of initial rate measurements, the rate constant $k_4 = 0$. According to the mechanism (Gutfreund & Sturtevant, 1956; Segel, 1975) given in eq 1, the kinetic parameters k_{cat} and K_m obtained from the steady-state rate equation

$$v = k_{cat}[E]_0[S_0]/(K_m + [S_0]) \quad (2)$$

are given by

$$k_{cat} = \frac{k_3 k_5}{k_3 + k_5} \quad (3)$$

and

$$K_m = \frac{k_5(k_2 + k_3)}{k_1(k_3 + k_5)} \quad (4)$$

If $k_2 \gg k_3$, then

$$K_m = \frac{k_2 k_5}{k_1(k_3 + k_5)} \quad (5)$$

and $k_{cat}/K_m = k_3/K_S$, where $K_S = k_2/k_1$, the true equilibrium binding constant. For the case where hydrolysis of the thioester is rate determining for glutamine hydrolysis, if $k_3 \gg k_5$, then k_{cat} will equal k_5.

times lower than in the wild-type holoenzyme, and 10 times lower than in the mutant enzyme at equivalent concentrations of substrate (Lusty, 1992). An increase in the rate of thioester hydrolysis (k_5) relative to its formation (k_3) must be invoked to account for the observed steady-state concentration of the thioester intermediate with the small subunit (Lusty, 1992). Assuming $k_5 > k_3$ suggests that the rate-determining step of glutamine hydrolysis catalyzed by the uncomplexed subunit is the formation rather than hydrolysis of the thioester intermediate.

Inactivation by L-*2-Amino-4-oxo-5-chloropentanoate.* The large increase in the K_m for glutamine and the lowered steady-state levels of the thioester intermediate in the Lys841 enzyme could signify either a decreased affinity of the enzyme for the substrate (that is, an increase in the dissociation constant (K_S)) or a slower rate of conversion of the ES complex to the thioester intermediate. The evidence obtained from the kinetics of γ-glutamyl thioester formation indicated that the glutamine hydrolytic reaction proceeded through the formation of a thioester intermediate, but neither k_3 nor k_5 appeared to be significantly affected by the mutation in the Lys841 enzyme.

To examine this point further, mutant and wild-type enzymes were analyzed for their ability to react with 2-amino-4-oxo-5-chloropentanoate, a chloroketone analog of glutamine. Pinkus and Meister (1972) have shown that the chloroketone binds specifically and irreversibly to the active site of the glutaminase subunit of the *E. coli* complex, blocking glutamine utilization. The covalent enzyme–inhibitor complex formed by the reaction of the chloroketone with the active site cysteine residue (eq 6) is analogous to that of the γ-glutamyl intermediate normally formed in step 2 of the glutaminase reaction mechanism (eq 1).

Reaction 2

$$E + CK \underset{k_2'}{\overset{k_1'}{\rightleftharpoons}} E{\cdot}CK \xrightarrow[Cl^-]{k_i'} E\text{–}R \qquad K_I = \frac{k_2'}{k_1'} \tag{6}$$

According to the mechanism shown above, E·CK represents the reversible enzyme inhibitor complex, and E–R represents the covalent enzyme–4–oxonorvaline derivative. The rates of inactivation ($k'_{i,app}$) by the chloroketone of wild-type and mutant carbamyl phosphate synthetases were determined as described by Anderson et al. (1986) and Kitz and Wilson (1962) by assaying the glutaminase activity remaining at different times following the addition of the chloroketone. As shown in Figure 3A, the glutaminase activity of the wild-type holoenzyme is rapidly inhibited by the chloroketone. The values of K_I (the dissociation constant for the reversible EI complex) and of k_i' (the first-order rate constant for the formation of the covalent inhibitor complex) determined from these data are 3.3 ± 1.7 mM and 1.7 ± 0.3 min^{-1}, respectively (panel B). A rapid inactivation of glutaminase activity with similar first order kinetics follows the addition of the inhibitor of the isolated glutaminase subunit (panel C). In the case of the glutaminase subunit, howoever, a reciprocal plot of

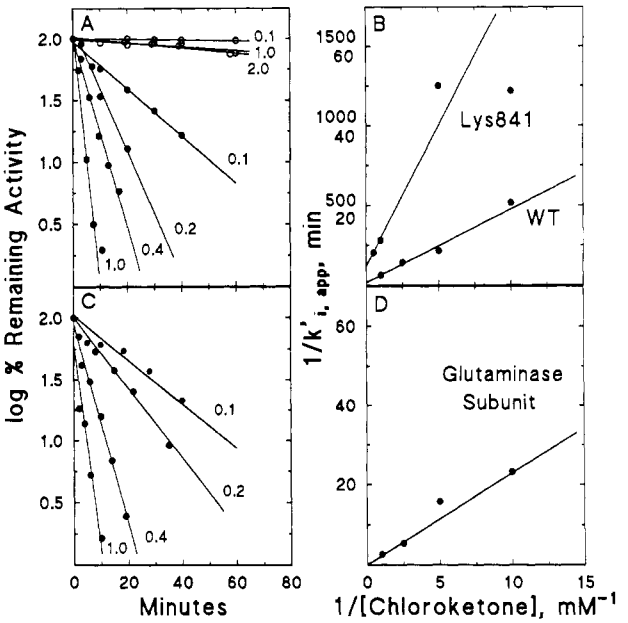

FIGURE 3: Inhibition of wild-type and Lys841 enzymes by 2-amino-4-oxo-5-chloropentanoate (chloroketone). Wild-type and mutant enzymes (12.3 μM) in 0.10 M potassium phosphate, pH 7.8, were incubated, at 37 °C, with the chloroketone at the indicated concentrations (millimolar). At the times shown, aliquots (25 μg) of the enzyme were removed, and glutaminase activity was assayed in the presence of 30 mM glutamine. (A) First-order plot of inactivation of wild-type (●) and mutant (○) enzymes. (B) Double-reciprocal plot of $k'_{i,app}$ versus chloroketone concentration. The lower range of values on the ordinate corresponds to $k'_{i,app}{}^{-1}$ of the wild-type enzyme, and the higher range to the mutant enzyme. (C) Inactivation of wild-type glutaminase subunit (12 μM) at four different concentrations (millimolar) of the chloroketone. (D) Dependence of $k'_{i,app}$ on the concentration of inhibitor. The values of $k'_{i,app}$ were calculated by least-squares fitting of the data shown in the first-order plots of (A) and (C) to the equation for single-exponential decay.

$k'_{i,app}$ versus chloroketone concentration describes a straight line that intercepts the axis at the origin (panel D). This behavior indicates that the glutaminase subunit forms only a weak reversible E·I complex with the chloroketone, an observation consistent with the apparent lower affinity of the small subunit for glutamine. Because K_I for the chloroketone is large compared to the range of inhibitor concentrations that could be used in the experiment, $k'_{i,app}$ appears to go through the origin. Nonetheless, the pseudo-first-order rate constant, estimated from the slope of the plot at 1 mM chloroketone (Figure 3C), is 0.46 min^{-1}, compared with the value of 0.40 min^{-1} established for the holoenzyme under the same conditions (panel A). Assuming these rates of inactivation to be similar and to reflect the rate of acylation, it is reasonable to conclude that the rate constant k_3 in the glutaminase reaction mechanism (eq 1) is also similar for the two proteins.

In contrast to wild-type holoenzyme and the glutaminase subunit, the reaction of the Lys841 enzyme with the chloroketone analog is much slower, even at high concentrations of inhibitor. For example at 2 mM chloroketone, the $t_{1/2}$ of inactivation of the mutant enzyme is 142 min. At the same concentration of inhibitor, the loss of glutaminase activity in the wild-type enzyme was too rapid to be measured accurately (Figure 3A). Plots of ln $k'_{i,app}$ against the ln of the chloroketone concentration gave slopes of 0.98, 1.05, and 0.80 for the wild-type complex, glutaminase, and Lys841 enzyme, respectively, suggesting that the loss of activity is caused by a reaction of the chloroketone with a single group on the

[4] The amount of thioester formed as a function of glutamine concentration (Figure 2) was analyzed by fit of the experimental data to the equation of Fersht (1985)

$$[NH_3] = [TE]_{ss} = \frac{[E_0]k_3[S]/(K_S + [S])}{k_5 + k_3[S]/(K_S + [S])} \tag{7}$$

where k_3/K_S is equal to k_{cat}/K_m, the second-order rate constant for overall acylation. In the analysis shown here, k_{cat} was expressed as $k_3k_5/(k_3 + k_5)$.

enzyme. The values of K_I and of k_i' determined from the data shown in Figure 3B are 1.7 ± 0.3 mM and 0.01 ± 0.001 min^{-1}, respectively. K_I appears comparable to that of the wild-type protein. The 170-fold decrease in the first-order rate constant, k_i', on the other hand, indicates that the mutation leads to either a change in the positioning, or a reduction in the nucleophilicity of the active site cysteine residue.

The inhibition by chloroketone indicates that the Lys841 substitution has only a marginal effect on binding of the glutamine analog. This result is in contradiction to the observed increase in K_m of the enzyme for glutamine. A second discrepancy is the decreased inhibition by the chloroketone even though the k_{cat} for glutamine hydrolysis is not changed as a result of the mutation. Both apparent discrepancies might be explained if the glutaminase rate is limited at the thioester hydrolytic step (k_5) or by the release of enzyme-bound glutamate. This would account for the similar K_I for the glutamine analog in both enzymes since this constant reflects a true equilibrium binding step. The K_m, however, which also includes the rate constants for thioester formation would be expected to increase in the mutant. The rate-determining hydrolysis step would not apply to the reaction with the chloroketone, since the reaction only goes as far as the SH group (comparable to thioester formation) and is irreversible. Thus, the interaction with the synthetase subunit may influence binding (K_I), but the mutation has no effect, whereas the reverse is true with respect to the rate of the reaction with the SH group.

Lack of Glutaminase Stimulation by ATP and Bicarbonate in the Mutant Enzyme. One of the clearest indications of the catalytic site interactions in carbamyl phosphate synthetase is the stimulation of the basal glutaminase actiivty by MgATP and HCO_3^- (Anderson & Meister, 1966). Since bicarbonate and both ATP and Mg^{2+} are required, the stimulation has been presumed to be caused by a carboxyphosphate-induced conformational change in one or both subunits. The glutaminase activity of mammalian pyrimidine-specific carbamyl phosphate synthetase (CAD) has also been reported to be stimulated when the enzyme is engaged in carboxyphosphate synthesis (Chaparian & Evans, 1991). The enhancement in the glutaminase catalytic rate has been inferred to be due to increases in the rate constants k_3 and k_5 (steps 2 and 3 in eq 1) leading to a more rapid conversion of enzyme-bound glutamine to glutamic acid and NH_3 (Chaparian & Evans, 1991).

Alterations of an interactive Cys-His ion pair (see discussion below) as indicated by the observed 200 times slower rate of reaction with the chloroketone in the mutant might however be reflected in loss of the normal enhancement of the glutaminase rate by carboxyphosphate. This was tested by measuring the effect of MgATP and bicarbonate on hydrolysis of glutamine and of γ-glutamyl hydroxamate. γ-Glutamyl hydroxamate is as efficient a substrate for the glutaminase as glutamine and has the advantage that its hydrolysis does not lead to formation of a derivative of either carbamate or carbamyl phosphate. A second advantage of using γ-glutamyl hydroxamate is the absence of stoichiometric cleavage of ATP that occurs with glutamine (Anderson & Meister, 1966). The use of this analog therefore avoids possible complications in interpreting kinetic data arising from other reaction intermediates.

At saturating concentrations, wild-type and mutant enzyme hydrolyze glutamine with nearly the same rates (Table III). This is also true of γ-glutamyl hydroxamate, although the K_m for this substrate analog was not measured. Addition of

Table III: Effect of MgATP and Bicarbonate on Glutamine and γ-Glutamyl hydroxamate Hydrolysis

enzyme activity	specific activities μmol/(min·mg) wild-type enzyme	Lys841 enzyme	isolated glutaminase
glutamine hydrolysis[a]			
minus MgATP and HCO_3^-	0.013	0.017	0.054
MgATP (2 mM), HCO_3^- (20 mM)	0.787	0.009	0.026
MgATP (2 mM), HCO_3^- (200 mM)	0.785	0.008	nd[c]
γ-glutamyl hydroxamate hydrolysis[b]			
minus MgATP and HCO_3^-	0.028	0.025	
MgATP (5 mM), HCO_3^- (20 mM)	5.50	0.010	
MgATP (5 mM), HCO_3^- (100 mM)	4.45	0.013	

[a] Conditions: 37 °C, pH 7.5, 50 mM Hepes/NaOH, 100 mM KCl, 15 mM $MgCl_2$, 30 mM glutamine. [b] Conditions: 37 °C, pH 7.5, 50 mM Hepes/NaOH, 100 mM KCl, 15 mM $MgCl_2$, 5 mM γ-glutamyl hydroxamate. [c] nd: not determined.

MgATP and HCO_3^- increases the rate of glutamine and γ-glutamyl hydroxamate hydrolysis by wild-type carbamyl phosphate synthetase by 60- and 200-fold, respectively. In the presence of MgATP and HCO_3^-, we calculate a turnover of glutamine hydrolysis by the wild-type enzyme of about 10 s^{-1} with a catalytic efficiency (k_{cat}/K_m) of 6.2 × 10^4 s^{-1} M^{-1}.

The addition of MgATP and HCO_3^- fails to elicit any increase in the hydrolysis of either glutamine or γ-glutamyl hydroxamate by the mutant enzyme. In fact, addition of bicarbonate and MgATP decreased the rate to half of that seen in their absence. In this respect, the mutant enzyme is similar to the isolated glutaminase whose hydrolytic rate is also reduced by inclusion of Mg^{2+}, ATP, and bicarbonate in the assay (Table III). The maximal catalytic activity of the Lys841 enzyme in glutamine hydrolysis is about 0.1 s^{-1}, or 100 times less than measured in the wild type. Combined with the 60 times lower K_m for glutamine, the overall catalytic efficiency (k_{cat}/K_m) of the Lys841 enzyme in glutamine hydrolysis is about 10 s^{-1} M^{-1}, a value 6000 times less than measured in the wild type. This greatly reduced hydrolytic rate is separate from any effect of the mutation on carboxyphosphate utilization (Guillou et al., 1992).

pH Dependence of Glutaminase Activity. The marked reduction in catalytic activity and in the rate of inhibition of the Lys841 enzyme by the chloroketone analog of glutamine points to an effect of the mutation on the charge properties of essential catalytic groups in the glutaminase active site. Therefore, it was of interest to examine the pH dependence of glutamine hydrolysis catalyzed by the mutant and wild-type enzymes. The glutaminase activity of wild-type carbamyl phosphate synthetase displays two pH optima; one at pH 4.3 and the other at pH 9.3 (Figure 4A and Trotta et al. (1973)). A third optimum is discerned as a shoulder on the main peak in the alkaline part of the range. This complex profile is altered to a single optimum (pH 6.6) in the Lys841 enzyme (Figure 4B). The pH dependence of the Lys841 enzyme is almost identical to that reported for the purified glutaminase (Trotta et al., 1973). The two apparent pKs (pK_{es} ~5.2 and pK_{es} ~8) seen in the pH profiles of the isolated glutaminase and of the mutant complex (Figure 4B) are consistent with the pKs of cysteine and histidine. Both residues have been suggested to participate in the formation of an interactive thiol–imidazolium ion pair (Amuro et al., 1985; Chaparian & Evans, 1991; Miran et al., 1991) analogous to that reported to exist in papain (Baker & Drenth, 1987; Polgar & Halasz, 1982).

The difference in the pH curves of the free and complexed glutaminase has been rationalized in terms of the presence in the glutaminase active site of amino acid side chains originating

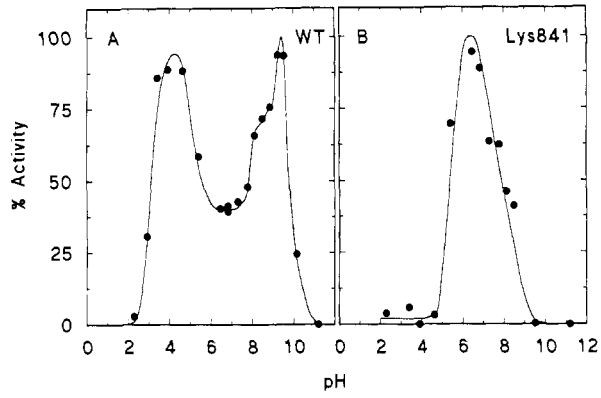

FIGURE 4: pH dependence of glutaminase activity. Reaction mixtures (0.20 mL) contained 50 mM glutamine in the following buffers: 0.1 M H_3PO_4/potassium phosphate, in the range pH 2.3–3.2; 0.1 M acetic acid/NaOH, from pH 3.4 to 4.0; 0.1 M acetic acid/K_2HPO_4, in the range pH 4.6 to 6.5; 0.1 M potassium phosphate, from pH 6.8 to 7.3; 0.1 M sodium barbital/KH_2PO_4, at pH 7.8; and 0.1 M sodium carbonate/KH_2PO_4, in the range pH 8.1–11.2. In (A), glutaminase activity is expressed as percent of the activity measured at pH 9.35 (100% = 73 nmol/(min·mg)). In (B), percent activity is expressed as percent of the activity obtained at pH 6.6 (55 nmol/(min·mg)). The pH values of the reaction mixtures were measured at 37 °C. The apparent pK_a values of the Lys841 enzyme substrate complex were estimated from plots of log V versus pH.

from the synthetase backbone (Trotta et al., 1973). An alternative plausible explanation is that conformational changes transmitted through the synthetase alter the composition of titratable groups in the vicinity of the proposed thiol–imidazolium ion pair in the active site of the glutaminase. Whatever the correct explanation, it is obvious that, notwithstanding the physical association of the two subunits in the mutant complex, the glutamine hydrolytic site is no longer responsive to the catalytic events occurring in the synthetase subunit.

Glu841 provides the first instance of a residue that not only abolishes an important reaction catalyzed by the synthetase subunit but exerts an equally profound effect on the kinetic and substrate-binding properties of the glutaminase active site. A direct participation of the glutamic acid residue in hydrolysis of the thioester intermediate seems unlikely in view of the ability of the isolated glutaminase subunit to catalyze hydrolysis of the thioester. The altered properties of the glutaminase in the mutant enzyme rather suggest a distortion in the disposition of key active site residues such as cysteine and histidine. The resultant apparent loss of catalytic coupling of the two subunits strongly argues for the proximity of Glu841 to the glutaminase active site. This in turn suggests the possibility of a direct physical contact between the carbamate phosphorylating domain in the carboxyl half of the synthetase subunit and the active site of the glutaminase subunit.

REFERENCES

Amuro, N., Pulah, J. L., & Zalkin, H. (1985) *J. Biol. Chem. 260*, 14844–14849.

Anderson, P. M. (1977) *Biochemistry 16*, 587–592.

Anderson, P. M., & Meister, A. (1966) *Biochemistry 5*, 3157–3163.

Anderson, P. M., & Carlson, J. D. (1975) *Biochemistry 14*, 3688–3694.

Anderson, P. M., Johnson, W. V., Endrizzi, J. A., Little, R. M., & Korte, J. J. (1987) *Biochemistry 26*, 3938–3943.

Baker, E. N., & Drenth, J. (1987) in *Biological Macromolecules and Assemblies, Volume 3, Active Sites of Enzymes* (Jurnak, F. A., & McPherson, A., Eds.) pp 314–367, John Wiley & Sons, New York.

Brent, E., & Bergmeyer, H. U. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Ed.) pp 1704–1708, Academic Press, New York.

Chaparian, M. G., & Evans, D. R. (1991) *J. Biol. Chem. 266*, 3387–3395.

Fersht, A. (1985) in *Enzyme Structure and Mechanism*, 2nd ed., pp 134–135, W. H. Freeman, New York.

Guillou, F., Liao, M., Garcia-Espana, A., & Lusty, C. J. (1992) *Biochemistry 31*, 1656–1664.

Gutfreund, H., & Sturtevant, J. M. (1956) *Biochem. J. 63*, 656–661.

Kitz, R., & Wilson, I. B. (1962) *J. Biol. Chem. 237*, 3245–3249.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Leatherbarrow, R. J. (1987) *Enzfitter*, Elsevier-Biosoft, Hills Road, Cambridge, U.K.

Lusty, C. J. (1992) *FEBS Lett. 314*, 135–138.

Meister, A. (1989) *Adv. Enzymol. Relat. Area Mol. Biol. 62*, 315–374.

Miran, S. G., Chang, S. H., & Raushel, F. M. (1991) *Biochemistry 30*, 7901–7907.

Nyunoya, H., & Lusty, C. J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4629–4633.

Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891–2899.

Piette, J., Nyunoya, H., Lusty, C. J., Cunin, R., Weyens, G., Crabeel, M., Charlier, D., Glansdorff, N., & Pierard, A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4134–4138.

Pinkus, L. M., & Meister, A. (1972) *J. Biol. Chem. 247*, 6119–6127.

Polgar, L., & Halasz, P. (1982) *Biochem. J. 207*, 1–10.

Post, L. E., Post, D. J., & Raushel, F. M. (1990) *J. Biol. Chem. 265*, 7742–7747.

Raushel, F. M., & Villafranca, J. J. (1980) *Biochemistry 19*, 3170–3174.

Rubino, S. D., Nyunoya, H., & Lusty, C. J. (1986) *J. Biol. Chem. 261*, 11320–11327.

Segel, I. H. (1975) in *Enzyme Kinetics*, pp 544–560, John Wiley & Sons, New York.

Trotta, P. P., Burt, M. E., Haschemeyer, R. H., & Meister, A. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 2599–2603.

Trotta, P. P., Wellner, V. P., Pinkus, L. M., & Meister, A. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2717–2721.

Trotta, P. P., Pinkus, L. M., Haschemeyer, R. H., & Meister, A. (1974) *J. Biol. Chem. 249*, 492–499.

Wellner, V. P., Anderson, P. M., & Meister, A. (1973) *Biochemistry 12*, 2061–2066.