BIOORGANIC CHEMISTRY 14, 163–169 (1986)

Inhibition of *Escherichia coli* Glutamine Synthetase by Phosphinothricin[1]

JOHN A. COLANDUONI AND JOSEPH J. VILLAFRANCA[2]

Department of Chemistry, Pennsylvania State University, University Park, Pennsylvania 16802

Received November 1, 1985

The inhibition of *Escherichia coli* glutamine synthetase by phosphinothricin [2-amino-4-(methylphosphinyl)butanoic acid] has been studied. This amino acid was observed to function as an active site directed inhibitor exhibiting time-dependent inhibition of glutamine synthetase in the presence of ATP or adenylylimidodiphosphate (AMPPNP) but not adenylyl(β,γ-methylene) diphosphonate (AMPPCP). The inactivation was observed to be pseudo-first order. Phosphinothricin was also found to inhibit the enzyme reversibly under initial rate conditions and was competitive with respect to glutamate with $K_{IS} = 18 \pm 3$ μM. The inactive enzyme inhibitor complex was found to contain approximately 11 molecules of ADP and of ^{32}P per dodecamer using [γ-^{32}P]ATP. Reactivation of the inactive enzyme complex was achieved by incubating the enzyme complex in 50 mM acetate (pH 4.4), 1 M KCl, and 0.40 M $(NH_4)_2SO_4$. ADP, phosphinothricin, and P_i were released upon reactivation.

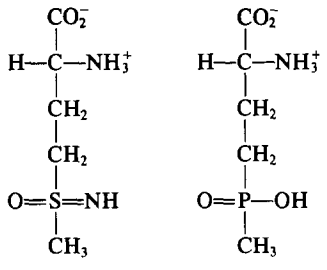
INTRODUCTION

The mechanism of the glutamine synthetase reaction has been explored by several research groups (*1–7*). The initial step of the reaction involves formation of γ-glutamyl phosphate from ATP and glutamate and this reaction has been studied by many independent methods (*4–7*). The second step of the reaction involves attack by NH_3 on γ-glutamyl phosphate to form a tetrahedral adduct. Several compounds have been studied that mimic the tetrahedral center of this adduct (*8–11*) and all have been found to bind tightly to glutamine synthetase isolated from several sources (*11–13*). Of these compounds methionine sulfoximine (**1**) is known to be phosphorylated (*14, 15*) and forms a very tight complex with the enzyme that can be released upon lowering the pH in the presence of high salt (*16*). Phosphinothricin (**2**) [2-amino-4-(methylphosphinyl)butanoic acid] is a potent inhibitor of glutamine synthetase (*10, 11*) that has been suggested to be an irreversible inhibitor under certain conditions. It was the purpose of this study to explore the kinetics and stoichiometry of inhibition of *Escherichia coli* glutamine synthetase by phosphinothricin. Our studies suggest that phosphinothricin inhibits the enzyme in a manner directly analogous to that found with methionine sulfoximine.

[1] This work was supported in part by an NIH grant (GM 23529).

[2] To whom correspondence should be addressed.

0045-2068/86 $3.00



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MATERIALS AND METHODS

Glutamine synthetase was prepared from *E. coli* cells grown in a nitrogen limiting medium. The method for isolating the enzyme from the cells followed the procedure of Miller *et al.* (*17*). This procedure utilizes the ability to precipitate glutamine synthetase with Zn^{2+}. The adenylylation state and concentration of the enzyme were determined spectrophotometrically. The adenylylation state was determined to be 2.7 (*18*). The concentration of enzyme is based on a subunit molecular weight of 50,000 for all calculations.

Phosphinothricin was a gift from Hoechst Chemical Company. Adenylyl-imidodiphosphate (AMPPNP) and adenylyl(β,γ-methylene)-diphosphonate (AMPPCP) were purchased from Boehringer-Mannheim Biochemicals. All other nucleotides, enzymes, substrates, and inhibitors were purchased from Sigma Chemical Company.

The biosynthetic activity was monitored using the lactate dehydrogenase and pyruvate kinase coupling system as described in earlier work (*9*). Where saturated the substrates were 50 mM NH_4Cl, 200 mM L-glutamate, and 2 mM ATP. Assays were performed in 1.0-cm cuvettes with 1.00 ml total volume in a Beckman DU spectrophotometer equipped with a Gilford attachment at 25°C at 339 nm. Assays were initiated with 10-μl aliquots containing 1.5 to 10 μg of glutamine synthetase.

Experiments monitoring the rate of inactivation of glutamine synthetase with phosphinothricin were conducted by the addition of 2 mM ATP (first) and 10 μM phosphinothricin (second) to 1.3 μM glutamine synthetase in 50 mM Hepes (pH 7.50), 100 mM KCl, and 15 mM $MgCl_2$ at 25°C. Aliquots of this mixture were removed and assayed for biosynthetic activity at various time intervals. Experiments designed to study the protection from inactivation were performed under identical conditions except substrates or inhibitors were present in the incubation mixture.

Phosphinothricin was also tested as a competitive inhibitor under initial rate conditions with glutamate as the varied substrate in the biosynthetic activity assay as described above. Conditions are identical to those of the standard assay except that the phosphinothricin and glutamate concentrations were varied.

The stoichiometry of adenine nucleotide bound to the inactive complex was

determined by incubating 130 μM glutamine synthetase with 2 mM phosphinothricin and 2 mM ATP in 50 mM Hepes (pH 7.50), 100 mM KCl, and 15 mM $MgCl_2$. The incubation mixture was allowed to react for 6 h at 25°C and was then assayed for activity to ensure complete inactivation. The enzyme was then dialyzed twice for 12 h each in the same buffer as used in the incubation experiment to allow dilution of unreacted ATP and phosphinothricin. The uv–vis spectrum was obtained and the stoichiometry determined by the same method used to determine the state of adenylylation of the enzyme (*18*).

The stoichiometry of the γ-phosphate of ATP that was bound to the inactive enzyme was determined by the addition of 2 mM [γ-^{32}P]ATP (1021 cpm/μmol) and 2 mM phosphinothricin to 130 μM of glutamine synthetase in the same buffer used above. The total volume was 1.0 ml. The enzyme was then assayed for activity to ensure complete inactivation. The enzyme was then dialyzed in the same buffer twice for 12 h each. The amount of radioactivity was then evaluated using a Beckman 7500 liquid scintillation counter.

Reactivation of the enzyme–inhibitor complex was performed by adding 0.40 ml of inactivated enzyme (520 μM) to 3.60 ml of 50 mM acetate (pH 4.1), 1 M KCl, and 0.40 M $(NH_4)_2SO_4$ at 25°C (*16*). The increase in activity was followed at various times as mentioned above.

The reactivation mixture was then lyophilized and 2.0 ml of deuterated water was added. The ^{31}P spectrum was taken on a Bruker 360-MHz NMR. Control experiments with ATP, ADP, and P_i were performed under identical conditions.

The reactivation mixture in deuterated water was also analyzed for inorganic phosphate by the spectrophotometric method of Penney using molybdic acid and malachite green (*19*).

RESULTS

Reversible Inhibition of Glutamine Synthetase by Phosphinothricin

Phosphinothricin was tested as a reversible inhibitor of glutamine synthetase under initial rate conditions with glutamate as the varied substrate. The inhibition was competitive with glutamate and a K_{IS} of 18 ± 3 μM was obtained where K_{IS} is defined as the binding constant of phosphinothricin to the enzyme–ATP complex.

Inactivation of Glutamine Synthetase by Phosphinothricin

When glutamine synthetase was incubated with phosphinothricin and ATP, a time-dependent inactivation of the enzyme was observed. The rate of inactivation could only be followed at extremely low phosphinothricin concentrations (<20 μM) since the inactivation was extremely fast. Therefore the enzyme concentration had to be very low for first-order behavior to be observed. The extremely fast turnover for inactivation together with the fact that phosphinothricin binds tightly to the enzyme made the normal concentration-dependent behavior studies difficult. Therefore the rate of inactivation at saturating phosphinothricin concentrations had to be estimated from the rate at a single subsaturating concentration of

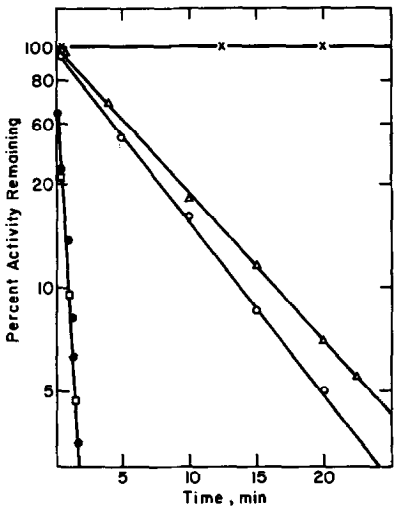

FIG. 1. Time course of inactivation of glutamine synthetase by phosphinothricin. Solutions contained 10 μM phosphinothricin alone (×) or phosphinothricin plus 2 mM ATP (●). In additional experiments, solutions contained ATP and phosphinothricin plus either 100 mM Glu (△), 1.0 mM 2-amino-4-phosphonobutyric acid (○), or 50 mM NH_3 (□). See Materials and Methods for additional experimental details.

phosphinothricin while the concentration of ATP was kept saturating at 2 mM. The results are shown in Fig. 1 for 10 μM phosphinothricin and 1.3 μM glutamine synthetase. The observed rate constant for inactivation, k_{obs}, under these conditions is 0.025 s^{-1}. The mechanism used to analyze the data was

$$E + I \overset{K_a}{\rightleftharpoons} EI \overset{k_1}{\rightarrow} EI^* \qquad [1]$$

where E is the enzyme, I is phosphinothricin, EI is the Michaelis complex, EI* is the inactive modified enzyme, K_a is the dissociation constant of phosphinothricin from the enzyme–ATP complex, and k_1 is the turnover rate for inactivation. Analysis of this type of mechanism yields the relationship

$$1/k_{obs} = (K_a/k_1)(1/[I]) + 1/k_1. \qquad [2]$$

Assuming that the K_{IS} value is equivalent to the K_a value, a k_1 value of 0.10 s^{-1} was determined.

Incubation experiments shown in Fig. 1 show that ammonia provides no protection from inactivation by phosphinothricin while 2-amino-4-phosphonobutyric acid (APBA) and glutamate exhibited approximately the same amount of protection. Also, phosphinothricin alone does not inactivate the enzyme.

The K_a value can also be calculated from the relative inactivation rates under conditions with and without glutamate (Fig. 1). Using k_{obs} = 0.025 s^{-1} (without glutamate) and $k_{obs'}$ = 0.0016 s^{-1} (with glutamate) and the equation

$$\frac{k_{obs'}}{k_{obs}} = \frac{[K_a/[I] + 1]}{[K_a'/[I] + 1]} \qquad [3]$$

K_a can be calculated independently using K_m = 3 mM for glutamate and $K'_a = K_a(1 + [Glu]/K_m)$. From these data, K_a = 10 ± 2 μM.

When phosphinothricin was incubated with AMPPCP at a concentration of 2 mM no inactivation of the enzyme was found. However, incubation of 2 mM phosphinothricin with 2 mM AMPPNP resulted in complete inactivation. In order to determine whether a small amount of ATP was contaminating the AMPPNP solution the inactivation experiment was conducted at 2 mM phosphinothricin and a twofold excess of AMPPNP over enzyme (20 μM AMPPNP with 10 μM glutamine synthetase). The enzyme was totally inactivated with a half-life of approximately 6 h while in a control experiment without AMPPNP the enzyme lost no activity under identical conditions.

Stoichiometry Experiments

Glutamine synthetase was incubated with phosphinothricin and [γ-^{32}P]ATP under conditions identical to those described above. After separation of the unreacted ATP, 10.6 ± 0.5 mol of ADP was found bound per dodecamer as determined by the increase in absorbance at 260 nm. The number of moles of ^{32}P from [γ-^{32}P]ATP was determined to be 11.3 ± 0.4 per dodecamer.

Reactivation Experiments

Glutamine synthetase (208 nmol) was inactivated with ATP and phosphinothricin under conditions described above. The inactive enzyme solution was then adjusted to pH 4.1 by addition of 50 mM acetate, 1.0 M KCl, and 0.40 M $(NH_4)_2SO_4$. Full reactivation was observed after 19 min. When AMPPNP was used instead of ATP (see above), full reactivation of the enzyme was also found.

The ^{31}P NMR spectrum of the solution obtained after reactivation showed peaks which were identified as ADP, P_i, and phosphinothricin. The amount of phosphate from this experiment was determined by chemical methods to be 190 ± 30 nmol as expected from the previous experiments used to determine the stoichiometry of inactivation.

DISCUSSION

From the kinetic data reported in this paper, the inactivation of *E. coli* glutamine synthetase by phosphinothricin proceeds via the formation of a reversible Michaelis complex between the enzyme, ATP, and phosphinothricin prior to the formation of the "irreversible" inactive complex. The binding constant for phosphinothricin is approximately 18 μM from the steady-state experiments where competitive behavior is found with respect to glutamate. Under similar conditions, glutamate binds to the enzyme with a K_d of approximately 3 mM in the presence of ATP (*20*). Since phosphinothricin is an amino acid with a tetrahedral phosphorus atom at the γ position, tight binding of this molecule to the enzyme is attributed to it being a mimic of the tetrahedral adduct formed in the normal reaction with glutamate, ATP, and NH_3. More interesting is the fact that this

inhibitor behaves like methionine sulfoximine (MSOX) in that it forms an inactive complex with the enzyme and ATP. This reaction can be contrasted to the reaction of another transition-state analog, 2-amino-4-phosphonobutyric acid, which binds to the enzyme but does not produce inactivation in the presence of ATP (J. Colanduoni and J. J. Villafranca, unpublished results). The only difference in the structures of phosphinothricin and APBA is that APBA does not contain a methyl group on the phosphorus atom but has another oxygen atom instead. When APBA reacts with ATP in the presence of enzyme, a phosphorylated product is formed with the phosphorylation taking place on the phosphorus moiety forming the diphosphate. The diphosphate is released from the enzyme and the catalytic reaction continues.

In the reaction of MSOX and ATP with glutamine synthetase, methionine sulfoximine phosphate and ADP are formed and are tightly bound to the enzyme resulting in inactivation (*13, 14–16*). The stoichiometry and ^{31}P NMR experiments with phosphinothricin show that ADP is bound to the inactive enzyme as is the γ-phosphate group of ATP. However, when the enzyme is reactivated, inorganic phosphate is released based on both the NMR and chemical determination experiments. Therefore if phosphinothricin is phosphorylated like APBA and MSOX, the product is either unstable under the conditions of reactivation or is never formed and the inactive complex consists of tightly bound ADP, P_i, and phosphinothricin. However, since phosphinothricin is a phosphinic acid derivative, the diphosphate product is expected to be hydrolytically unstable especially under the acidic conditions used in the reactivation experiments.

Evidently the requirement for ATP in the inactivation reaction is not absolute since AMPPNP plus phosphinothricin also inhibits the enzyme. AMPPNP must phosphorylate phosphinothricin but the reaction takes place at a much slower rate than would be expected since P–N cleavage occurs in this reaction. AMPPCP does not replace ATP in the inactivation reaction as expected and thus the transfer of a phosphoryl moiety is required for inactivation with phosphinothricin.

Reactivation of glutamine synthetase inactivated with MSOX or phosphinothricin in the presence of ATP (or AMPPNP) can be achieved by acidification and addition of ammonium sulfate under conditions of high ionic strength (*16*). Based on these experiments as well as the others described in this paper, we conclude that the mechanism for inactivation of glutamine synthetase is similar for both phosphinothricin and MSOX.

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