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Affinity Labeling of Escherichia coli Glutamine Synthetase by β,γ-Cr(III)$(H_2O)_4$ATP*

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Summary: The interaction of Escherichia coli glutamine synthetase with β,γ-Cr(III)$(H_2O)_4$ATP (CrATP) has been studied. This substitution inert nucleotide functioned as an active site directed irreversible inhibitor of glutamine synthetase in solutions containing 15 mM $MgCl_2$, 100 mM KCl, and 10 mM Pipes(pH 6.6). The inactivation reaction followed pseudo-first order saturation kinetics which demonstrated reversible binding of CrATP prior to the formation of inactive enzyme. CrATP was shown to be a competitive inhibitor versus MgATP. Also, significant protection was afforded by MgATP indicating that CrATP inactivates at the active site. Partial protection was afforded by glutamate or inorganic phosphate while inactivation was enhanced by Mn(II). The stoichiometry of CrATP incorporation was approximately one molecule per enzyme subunit, determined spectrophotometrically. Both the Δ and Λ isomers of CrATP bound to glutamine synthetase, but only the Λ isomer was an active site directed irreversible inhibitor.

Glutamine synthetase of Escherichia coli catalyzes the synthesis of glutamine from glutamate and ammonia with the concomitant hydrolysis of ATP to ADP and inorganic phosphate. The enzyme is composed of 12 identical subunits each with a molecular weight of 50,000. Each subunit has a single catalytic site and at least two distinct divalent metal ion binding sites designated as the n_1 and the n_2 sites. Metal ion binding to the high affinity n_1 site is responsible for maintaining the active conformation of the enzyme and may be involved in the binding of glutamate and methionine sulfoximine (MSOX), a transition state analog resembling glutamate. The n_2 metal ion site is associated with nucleotide binding.

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This paper describes the interaction of the 'substitution inert' β,γ-Cr(III)$(H_2O)_4$ATP (CrATP) complex with glutamine synthetase and demonstrates how CrATP acts as a active site directed irreversible inhibitor. Other active site directed reagents that have been reported include the affinity label 5'-p-fluorosulfonylbenzoyladenosine which reacts with a specific lysine residue,[1,2] and L-methionine sulfoximine (MSOX) plus ATP which react and produce a phosphorylated derivative of MSOX that remains tightly bound at the active site.[3,4]

Cr(III) nucleotides have been used to probe the mechanism of many nucleotide requiring enzymes[5,6] and have been demonstrated to act both as enzyme inhibitors and substrates. As substrates, they typically phosphorylate a second substrate forming an intermediate complex which can only slowly dissociate from the enzyme.

Four different isomers of CrATP exist in solution. There are two different screw sense isomers designated Λ and Δ and each of these isomers has two different conformations thought to be stabilized by hydrogen bonding. The two different conformational isomers readily racemize due to a low energy barrier for this interconversion but racemization of the screw sense isomers does not occur readily due to the necessity of breaking the Cr-O bond. Enzymes in which CrATP can act as a substrate generally use only one of the screw sense isomers. In this paper we report that only one of these screw sense isomers is an active site directed irreversible inhibitor of E. coli glutamine synthetase.

MATERIAL AND METHODS

Lactate dehydrogenase, pyruvate kinase, NADH, phosphoenolpyruvate(PEP), ATP, glutamate, NH_4Cl, KCl, $MgCl_2$, KH_2PO_4, and piperazine-N,N'-bis[2-ethanesulfonic acid] (Pipes) were obtained from Sigma. Methanesulfonic acid was abtained from Alfa. All other chemicals were reagent grade.

E. coli cells were grown on a limited ammonia medium according to the procedure of Miller et al.[7] Unadenylylated glutamine synthetase in a low state of adenylylation ($\bar{n}$=2.7) was purified by the zinc precipitation method.[7] Purified glutamine synthetase was stored as a precipitate in saturated $(NH_4)_2SO_4$ with 15 mM $MgCl_2$. A stock solution of glutamine synthetase was prepared for experiments by dialysis of the precipitated enzyme at 4° C against 10 mM Pipes(pH 6.6) containing 100 mM KCl, and 15 mM $MgCl_2$. The concentration of glutamine synthetase was measured by the spectrophotometric method.[8]

β,γ-Cr(III)$(H_2O)_4$ATP (CrATP) was prepared by following the method of Dunaway-Mariano and Cleland.[9]

Kinetic Assays: Glutamine synthetase activity for both inhibition and inactivation studies was measured spectrophotometrically using the pyruvate kinase-lactate dehydrogenase coupling system. A Beckman DU spectrophotometer equipped with a Gilford 2000 optical density converter and a 10 mV recorder was used to monitor the conversion of NADH to NAD at 340 nm. Enzyme activity was measured at 25°C in 1 mL cuvettes. The reaction mixture common to all glutamine synthetase activity measurements contained 50 mM Pipes buffer, pH 6.6, 100 mM KCL, 15 mM $MgCl_2$, 50 mM NH_4Cl, 100 mM glutamate, 0.5 mM PEP, 0.1 mM NADH, 0.3 mg/mL pyruvate kinase, and 0.3 mg/mL lactate dehydrogenase.

Reaction of Glutamine Synthetase and CrATP: Glutamine synthetase (5 μM subunits) was incubated with various concentrations of CrATP (from 32 to 260 μM) at 25°C in a reaction mixture containing 50 mM Pipes buffer(pH 6.6), 100 mM KCl, 15 mM $MgCl_2$. The CrATP stock solution was prepared in 50 mM Pipes buffer with KCl and Mg just prior to the start of the experiment. The inactivation reaction was initiated by the addition the the CrATP solution to the enzyme solution. For each measurement a control was performed without the addition of CrATP. To follow the inactivation, 20 μL aliquots were withdrawn at appropriate times and added to an assay tube (final volume equaled 1 mL) containing the above described assay mixture and 2.4 mM ATP. The dilution of CrATP plus the presence of ATP quenched the inactivation reaction.

Protection of glutamine synthetase from inactivation by CrATP was studied by adding either 2.5 mM ATP, 50 mM glutamate, 50 mM P_i, 100 mM excess Mg(II), or 0.25 mM Mn(II) to the inactivation mixture containing 65 μM CrATP. Appropriate controls were performed without CrATP present.

Inhibition Studies with CrATP: Various amounts of CrATP and ATP were added to the above describe assay mixture prior to the initiation of the reaction by a 10 μL addition of glutamine synthetase (0.25 mg/mL). The total volume of the assay mixture was 1.0 mL. The experiment was performed with varied levels of ATP at 25, 50, 100, 200, and 2000 μM with different fixed levels of CrATP at 0, 26, 52, 104, 207 μM.

Stoichiometry of Inactivated Enzyme: Glutamine synthetase (25 μM subunits) was incubated with 300 μM CrATP at 25°C for 6 hours in a reaction mixture containing 50 mM Pipes(pH 6.6), 100 mM KCl, 15 mM $MgCl_2$. This reaction mixture was then dialyzed for 12 hrs at 4°C against buffer. The stoichiometry of adenylyl groups which remained bound to the enzyme was determined spectrophotometrically.[1]

Determination of the Isomer Causing the Inactivation: In order to determine which isomer (Δ or Λ) of CrATP caused the inactivation of glutamine synthetase, 300 μM of CrATP was treated with 300 μM glutamine synthetase(subunit concentration) in 15 mM $MgCl_2$, 100 mM KCl, and 15 mM Pipes(pH 6.6) at 20 °C. After 20 min, the reaction was stopped by placing the sample in an ice bath and addition of 50 μL of 0.2 M HCl which lowered the pH to 5.5. A portion of the enzyme precipitated. After removing the precipitate by centrifugation, the unreacted CrATP was separated from the remaining enzyme by an amicon ultra filtration system using a YM-50 filter.

The quantitation of the different isomers in the filtrate was performed by the HPLC method of Gruys and Schuster[10] in which a reverse phase C-18 column was used with 10 mM methanesulfonic acid (pH 2.5 NaOH) as the mobile phase. A Waters HPLC system was used. The chromatogram of the filtered CrATP solution that had reacted with the enzyme was compared to the chromatogram of untreated CrATP.

RESULTS

Inactivation of Glutamine Synthetase By CrATP: When glutamine synthetase was incubated with CrATP as described in Materials and Methods, the loss of catalytic activity followed pseudo-first order kinetics for 95%

of the reaction. From semilog plots, the first order rate constant, k_{obs} was calculated. The inactivation of glutamine synthetase displayed saturation kinetics consistent with the mechanism described by equation 1.

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} EI^* \qquad (1)$$

where E is the enzyme, I is CrATP, EI is the Michaelis complex, and EI^* is the inactive modified enzyme. Analysis of this type of mechanism yields the relationship given in equation 2 where $K_I=k_{-1}/k_1$.

$$1/k_{obs} = (K_I/k_2)(1/[I]) + 1/k_2 \qquad (2)$$

A plot of $1/k_{obs}$ versus 1/[CrATP] is shown in Figure 1. From this plot the values of K_I and k_2 were determined to be 90 μM and 2.1×10^{-3} sec^{-1}, respectively.

Effect of Substrates and Metal Ions on the Inactivation of Glutamine Synthetase: The effects that different substrates and metal ions have on the observed inactivation rate constant is shown in Table I. ATP afforded the greatest amount of protection against inactivation. Glutamate and inorganic phosphate showed an intermediate amount of protection. Excess Mg(II) showed little protection, while Mn(II) enhanced the inactivation.

Kinetic Inhibition Patterns for CrATP versus ATP: The data from steady-state kinetics employing the biosynthetic reaction showed that CrATP

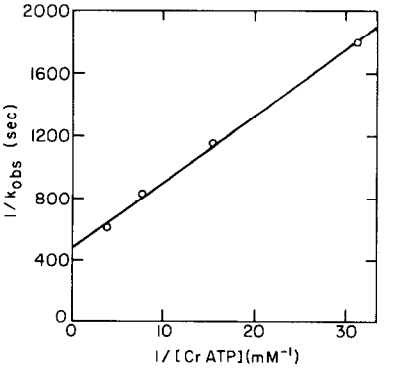

Figure 1. Double reciprocal plot for CrATP inhibition of glutamine synthetase. See Materials and Methods for experimental conditions.

Table I. Protection Against Inactivation with CrATP by Several Active Site Ligands. The first order rate constant for inactivation, k_{obs}, was measured under the incubation conditions given in Materials and Methods for each added ligand.

Ligand Added	k_{obs} (sec^{-1} x 10^{-3})
None	0.90
2.5 mM ATP	0.10
50 mM L-Glu	0.45
50 mM P_i	0.40
100 mM Mg(II)	0.83
0.25 mM Mn(II)	3.5

is a linear competitive inhibitor versus ATP. The relationship between the velocity and substrate concentration in the presence of a competitive inhibitor is given by equation 3.

$$1/v = (K_m/V_{max})(1+[I]/K_{is})(1/[S]) + 1/V_{max} \quad (3)$$

The data were fit using the programs of Cleland[11] and yielded the following values for the constants: K_{is}=33±7 μM, K_m=100±40 μM.

Stoichiometry of the Inactivation: The amount of CrATP which remained bound to inactivated glutamine synthetase after dialysis was determined spectrophotometrically. The increase in absorbance at 260 nm due to the adenylyl moiety of CrATP bound to the enzyme corresponded to the labeling of 93% of the enzyme subunits in enzyme that had lost >99% of its activity.

In order to determine if all four isomers of CrATP inactivate glutamine synthetase the enzyme was incubated with 1 and 2 equivalents of CrATP per monomer of enzyme. The results are shown in Figure 2. From these data, approximately half of the total CrATP is involved in the inactivation of the enzyme.

Determination of the CrATP Isomer which Caused the Inactivation of Glutamine Synthetase: Separation of the CrATP isomers by HPLC has been

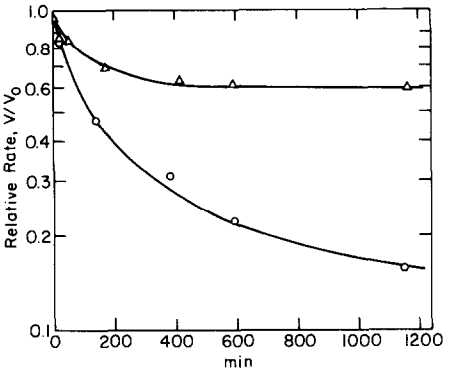

Figure 2. Inactivation with 1.0 and 2.0 equivalents of CrATP per monomer of glutamine synthetase. CrATP was kept at 24 μM in both inactivation experiments while glutamine synthetase was varied at 24 μM(Δ) and 48 μM(o). The incubation conditions and activity measurements were performed as described in Materials and Methods.

previously described by Gruys and Schuster.[10] A chromatogram shows four nearly resolved peaks, two peaks which correspond to the Δ isomer (peaks 1 and 4), and two peaks which correspond to the Λ isomer (peaks 2 and 3). The ratio of the peak areas of a control sample of CrATP was 51%(Δ isomer) to 49%(Λ isomer) given as percent of total area of the 4 peaks. A CrATP solution(equimolar with enzyme subunits, see Materials and Methods) which was incubated with glutamine synthetase to give inactive enzyme and then subjected to HPLC analysis had a ratio of Δ to Λ isomers of 91% to 9%. This result indicates that the Λ isomers must be responsible for the inactivation of glutamine synthetase.

DISCUSSION

From the kinetic data reported in this paper, the inactivation of *E. coli* glutamine synthetase by β,γ-CrATP proceeds via the formation of a reversible, Michaelis complex between the enzyme and CrATP prior to the formation of the 'irreversible' inactive complex. Attachment at or near the ATP binding site was demonstrated by the protection against inactivation afforded by MgATP. A possible pathway for the inactivation of glutamine synthetase may arise from a ligand substitution reaction. Thus, a water molecule on the substitution 'inert' Cr(III) ion may be displaced by an amino acid residue (X) at or near the ATP binding site resulting in the formation of a new 'stable' $Cr(H_2O)_3(X)ATP$

complex. The inactivation may proceed through a multi-step mechanism, however, the minimum scheme presented in equation 1 was sufficient to describe the observed behavior of the kinetic data.

The two to three-fold difference between the K_I determined from the inactivation experiment and the K_{is} from the kinetic experiments most likely reflects the slightly different experimental conditions, e.g., glutamate and NH_3 are absent in the inactivation experiments.

Analysis of the composition of CrATP isomers by HPLC leads to the conclusion that glutamine synthetase is inactivated by the Λ isomer(s) of CrATP. In considering the enironment of the n_2 metal-nucleotide binding site of glutamine synthetase one must remember that the metal ion and nucleotide can bind independently.[12,13,14] It is not surprising therefore that when levels of Mg(II) or Mn(II) are added to ensure saturation of the n_2 site(see Table I), an alteration occurs in the inactivation rate produced by CrATP. Magnetic resonance data are consistent with the simultaneous binding of CrATP and Mn(II) at the n_2 site[14] and our current experiments suggest that the Cr(III) moiety of the Λ isomer of enzyme-bound CrATP is positioned near an enzyme residue with which it can interact. From previous work in our laboratory,[15] the B isomer of Ado-5'-(2-thioPPP) was established as a substrate for glutamine synthetase in the presence of Mg(II). Thus, the stereochemistry of the chelate ring of the Λ isomer of β,γ-$Cr(H_2O)_4$ATP and the B isomer of Mg(II)-Ado-5'-(2-thioPPP) are identical. This correlation may mean that an amino residue which is a ligand for the Mg(II) could displace a water molecule of CrATP leading to inactivation by the formation of a new stable Cr(III) complex. Identification of the peptide and amino acid residue(s) that may be altered by reaction with CrATP is currently underway in our laboratory.

REFERENCES

1. Foster, W.B., Griffith, M.J., and Kingdon, H.S. (1981) J.Biol.Chem. 256 882-886

2. Pinkofsky, H.B., Ginsburg, K.A., Reardon, I., and Heinrikson, R.L. (1984) J.Biol.Chem. 259 9616-9622
3. Weisbrod, R.E., and Meister, A. (1973) J.Biol.Chem. 250 3505-3509
4. Rhee, S.G., Chock, P,B., Wedler, F.C., and Sugiyama, Y. (1981) J.Biol.Chem. 256 644-648
5. Dunaway-Mariano, D., and Cleland, W.W. (1980) Biochemistry 19 1506-1515
6. Dunaway-Mariano, D., Benovic, J.L., Cleland, W.W., Gupta, R.L., and Mildvan, A.S. (1979) Biochemistry 18 4347-4354
7. Miller, R.E., Shelton, E., and Stadtman, E.R. (1974) Arch.Biochem. Biophys. 163 155-171
8. Stadtman, E.R., and Ginsburg, A. (1974) The Enzymes, pp. 755-807, Academic Press
9. Dunaway-Mariano, D., and Cleland, W.W. (1980) Biochemistry 19 1496-1505
10. Gruys, K.J., and Schuster, S.M. (1983) Biochemistry 22 5237-5242
11. Cleland, W.W. (1967) Adv.Enzymol.Relat.Areas.Mol.Biol. 29 1-32
12. Hunt, J.B., Smyrniotis, P.Z., Ginsburg, A., and Stadtman, E.R. (1975) Arch.Biochem.Biophys. 166 102-124
13. Gibbs, E.J., Ransom, S.C., Cuppett, S., and Villafranca, J.J. (1984) Biochem.Biophys.Res.Commun. 120 939-945
14. Ransom, S.C. (1984) Ph.D. Thesis, Pennsylvania State University
15. Pillai, R.P., Raushel, F.M., and Villafranca, J.J. (1980) Arch.Biochem.Biophys. 199 7-15