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Affinity Labeling of *E. coli* GTP Cyclohydrolase I by a Dialdehyde Derivative of Guanosine Triphosphate

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Abstract: Time-dependent inactivation of *E. coli* GTP cyclohydrolase I with a 2′,3′-dialdehyde derivative of GTP (oGTP) was directed to the active site of the enzyme, and was dependent on the concentration of oGTP. The kinetics of inactivation were biphasic with a rapid reaction occurring immediately upon exposure of the enzyme to oGTP followed by a slow rate of inactivation. The K_i value of oGTP for the enzyme was 0.25 mM. Inactivation was prevented by preincubation of the enzyme with GTP, the substrate of the enzyme. At 100% inactivation, 2.3 mol of [8.5′-^{3}H]oGTP were bound per each enzyme subunit, which consists of two identical polypeptides. The active site residue which reacted with the affinity label was lysine. oGTP interacted selectively with the ε-amino group of lysine in the GTP-binding site to form a morpholine-like structure which was stable without sodium borohydride treatment. However, triphosphate group was eliminated during the hydrolysis step. To identify the active site of the enzyme, [8,5′-^{3}H]oGTP-labeled enzyme was cleaved by endoproteinase Lys-C, and the ^{3}H-labeled peptide was purified by HPLC. The amino acid sequence of the active site peptide was Pro-Ser-Leu-Ser-Lys, which corresponds to the amino-terminal sequence of the enzyme.

Key words: *E. coli*, GTP cyclohydrolase I, affinity label, dialdehyde derivative of GTP (oGTP).

GTP cyclohydrolase I (EC 3.5.4.16) catalyzes the conversion of GTP to dihydroneopterin triphosphate, the first step of the biosynthesis of folate in *E. coli* (Brown and Williamson, 1987). The reaction, which has been investigated in some detail, proceeds as at least a three-step process (Burg and Brown, 1968; Shiota *et al.*, 1967; Wolf *et al.*, 1969; Yim and Brown, 1976). The complex reaction starts with the release of formate from 8 carbon of the purine imidazole ring by two hydrolytic steps. The ribose group subsequently undergoes an Amadori rearrangement, followed by a ring closure involving the newly formed carbonyl group with the amino group at position 5 of the pyrimidine ring. The enzyme appears to be a tetramer of 210,000 MW which can be dissociated into identical subunits of 51,000 MW at relatively high salt concentrations. The enzyme, when dissociated into subunits, has little or no catalytic activity (Yim and Brown, 1976).

Various affinity labeling techniques have been used to study the active site structure of enzymes. Oxidation of ribonucleotides by periodate results in cleavage of the 2′,3′-diol to yield a dialdehyde derivative, but only recently the structure of the periodate-oxidized nucleotides was identified (Lowe and Beechy, 1982). Aldehyde derivatives are capable of reacting with primary amines, such as the ε-amino group of lysine and the α-amino group of the N-terminal amino acid. In this study the dialdehyde nucleotide analog was used to investigate the active site structure of *E. coli* GTP cyclohydrolase I in relation to the mechanism of enzyme catalysis.

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Materials and Methods

Materials

Escherichia coli B (ATCC 11303) was obtained from General Biochemicals. Sephadex G-200-120, Sephadex G-10-120, Sepharose-4B, GTP, sodium borohydride, sodium periodate, Trizma base, trypsin, α-chymotrypsin, lysozyme, DNase I, and endoproteinase Lys-C were purchased from Sigma. [8-^{14}C]GTP and [α-^{32}P]GTP were obtained from Amersham, and [8,5′-^{3}H]GTP was supplied by New England Nuclear. Toluene and triton X-100 were purchased from Merck. Proteinase K was supplied by Bio-Rad.

Purification of GTP cyclohydrolase I

The enzyme was purified from frozen *E. coli* cells

by the procedure of Yim and Brown (1976). The homogeneity of the enzyme was judged by SDS-polyacrylamide gel electrophoresis. The protein amount was estimated by the method of Bradford (1976).

Enzyme assays

Charcoal assay: This assay was based on the method developed by Burg and Brown (1968). The assay method measures release of the 8-carbon in the form of formate from [8-^{14}C]GTP. Appropriate amounts of the enzyme were added to solutions containing 100 μM GTP, 0.1 M Tris-HCl, 5 mM EDTA, and 0.1 M KCl, pH 8.0 in a final volume of 0.5 ml, and incubated at 42℃ for 30 min in the dark. The reaction was stopped by addition of 25 μl of 4 M formic acid. The radioactive formate liberated from [8-^{14}C]GTP was separated from residual radioactive compounds by treatment with charcoal. Since charcoal adsorbs unreacted GTP and H_2-neopterin-P_3, the amount of unadsorbed radioactivity represents the amount of formate from the substrate. Radioactivity was quantified in a Packard Tricarb model 1500 liquid scintillation counter.

Fluorescence assay: The method developed by Fukushima *et al.* (1977) was used, with some modifications. The standard reaction mixtures and conditions of incubation were identical to the charcoal assay. To quantify dihydroneopterin triphosphate formed from GTP, the enzyme reaction was stopped by addition of 25 μl of acidic iodine (5 N trifluoroacetic acid : 1% I_2-2% KI = 1 : 5). After 10 min, excess iodine was removed by addition of 25 μl of 2% ascorbic acid, followed by 25 μl of 1 N NaOH to bring the pH to 8.0. Fluorescence was measured using a Shimadzu RF-540 spectrophotometer (excitation at 360 nm, emission at 450 nm). One unit of enzyme activity was defined as the amount of enzyme which catalyzed the formation of 1 pmol of formate or dihydroneopterin triphosphate/min at 42℃.

Preparation of oGTP

oGTP was prepared by the method of Easterbrook-Smith *et al.* (1976). Sodium periodate (20 μmol) was added to 20 μmol of GTP dissolved in H_2O and the solution was allowed to stand at room temperature in the dark for 1 h. The reaction was stopped by the addition of ethylene glycol (10 μmol), and the reaction mixture was loaded onto a Sephadex G-10 column (1.2×50 cm) which had been previously equilibrated with water at 4℃. The column was eluted with water, and each fraction was tested for the presence of iodate, according to the method of Collier and Nishimura (1978). The fractions which did not contain iodate were pooled and lyophilized.

The concentration of oGTP was determined by measuring the absorbance at 260 nm, using an absorption coefficient value of 11,800 $cm^{-1} \cdot M^{-1}$ (Ball and Nishimura, 1980). The purity of oGTP was confirmed by thin layer chromatography. Thin layer chromatography was performed on polyethyleneimine sheets using 0.8 M NH_4HCO_3 as the developing solvent. The sheets were irradiated with UV light to localize the position of the nucleotide. The purified sample migrated as a single compound with an RF value of 0.01. No GTP (RF 0.53) was detected.

Treatment of the modified enzyme with sodium borohydride

GTP cyclohydrolase I (1.2 nmol) was incubated with 1.5 mM oGTP in 50 mM potassium phosphate buffer, pH 7.0, containing 5 mM EDTA at 42℃. After 1 h, sodium borohydride was added in a 20-fold excess over the amount of oGTP and the solution was allowed to stand for 30 min.

Identification of the modified amino acid

GTP cyclohydrolase I (4 nmol) was incubated with [8,5'-^{3}H] oGTP(5 mM) for 110 min at 42℃. After the reaction was stopped the solution was dialyzed extensively against water. The dialyzate was then lyophilized, redissolved in 0.25 ml of 0.1 M N-ethyl morpholine acetate buffer, pH 8.0, then the sample was subjected to enzyme digestion. The enzyme solution was first incubated for 3 hours with 0.2 mg of trypsin, followed by treatment with 0.2 mg of chymotrypsin (15 h), and finally 2 mg of proteinase K (24 h). Aliquots of the resulting digest were chromatographed by an ascending manner on Whatman 3 MM paper using 1-butanol/acetic acid/water (4/1/5, by volume) as the developing solvent. After drying, the sheet was cut into 1 cm sections and the radioactivity of each section was determined.

Lys-[8,5'-^{3}H]oGTP as a standard marker was prepared as follows: The reaction mixture contained, in a final volume of 0.2 ml, [8,5'-^{3}H]oGTP, 0.2 μmol; lysine, 0.6 μmol; Mg^{2+}, 0.3 μmol; and sodium borohydride, 0.6 μmol. The pH was adjusted to 11 using KOH. The mixture was allowed to stand at room temperature for 40 min. The adduct formed was purified by ascending paper chromatography on Whatman 3MM paper, using 1-butanol/acetic acid/water (4/1/5, by volume) as the developing solvent (Easterbrook-Smith *et al.*, 1976).

Amino acid analysis

The enzyme sample(5 nmol), which had been dialyzed against water, was lyophilized and the solid mate-

rial was dissolved in 30 μl of 6 N HCl. Hydrolysis was performed in a sealed evacuated glass tube at 110 ℃ for 24 h. After the HCl had been removed by evaporation, the hydrolysate was dissolved in 30 μl of 0.1 N HCl, and the material was analyzed on an amino acid analyzer (Waters model 510) using a cationic exchange amino acid column (0.38×30 cm).

Isolation and sequencing of the [^{3}H]-labeled peptide

GTP cyclohydrolase I (20 nmol), which had been inactivated by [8.5'-^{3}H]oGTP, was dialyzed against water, and cleaved by treatment with Endoproteinase Lys-C. Incubation was performed for 24 h at 37℃ in 0.2 ml of 80 mM Tris-HCl (pH 8.5) containing 3.2 M urea (Ogata *et al.*, 1992). The resulting material was subjected to reversed-phase HPLC on a Nova-pak C18 column (0.39×15 cm). The column was eluted with a linear gradient of 0 to 50% (v/v) acetonitrile in 0.1% trifluoroacetic acid. The eluates were monitored by absorbance at 214 nm, and the ^{3}H radioactivity contained in each fraction was determined. The major radioactivity peak was collected, then concentrated using a speed-vac apparatus. Approximately 150 pmol of the ^{3}H-labeled peptide was subjected to automated Edman degradation using a protein sequencer (Applied Biosystems Inc. Model 473A).

Results

Inactivation of GTP cyclohydrolase I by oGTP

The interaction between GTP cyclohydrolase I and oGTP was analyzed by incubating the enzyme (1.2 nmol) with 0.5 mM oGTP in 50 mM potassium phosphate buffer, pH 7.0, containing 5 mM EDTA at 42℃. At given times, the reaction was stopped by a 20-fold dilution of the reaction mixture, and the remaining enzyme activity was assayed. The results, shown in Fig. 1, show that the inactivation process is dependent on the presence of oGTP since a control system, in which oGTP was absent, did not lose enzyme activity upon incubation.

Samples of oGTP-inactivated enzyme, before and after borohydride addition, were subjected to dialysis and the enzyme activity was measured. The addition of borohydride had no effect on the rate or extent of inactivation. When the enzyme was inactivated by incubating with [8,5'-^{3}H]oGTP, and the material was subjected to Sephadex G-200 gel filtration, the radioactivity peak

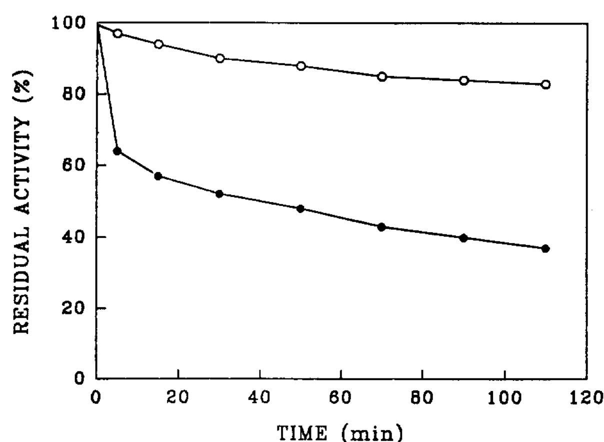

Fig. 1. Time course of the inactivation of GTP cyclohydrolase I by oGTP. 1.2 nmol of the enzyme in 50 mM KH_2PO_4 buffer (pH 7.0) containing 5 mM EDTA were incubated at 42℃ in the presence (●-●) and absence (○-○) of 0.5 mM oGTP and aliquots were assayed for activity at indicated time points.

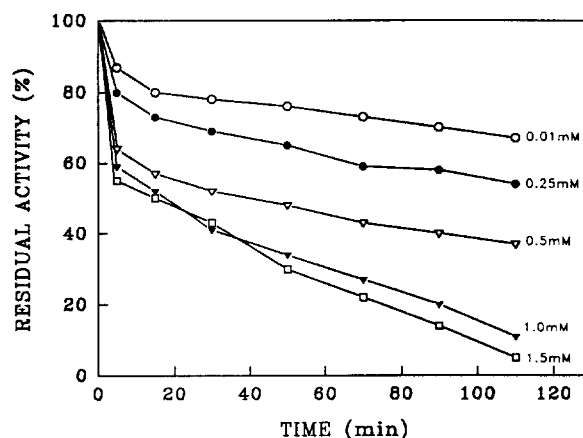

Fig. 2. Concentration dependence of oGTP on the rate of inactivation. 1.2 nmol of the enzyme in 50 mM KH_2PO_4 buffer (pH 7.0) containing 5 mM EDTA were incubated at 42℃. oGTP was then added to give final concentrations of 0.01, 0.25, 0.5, 1.0, and 1.5 mM. Aliquots were assayed for activity at the indicated times.

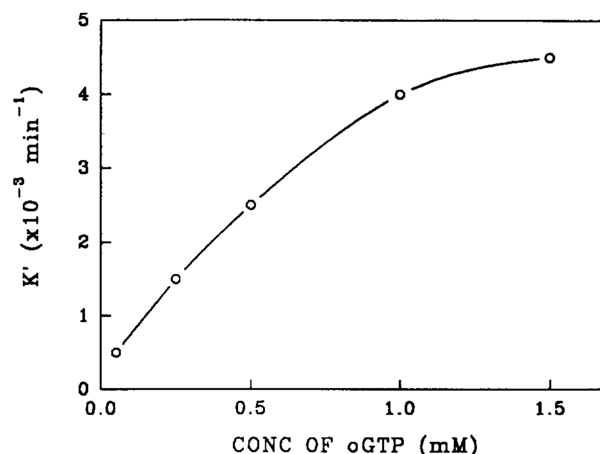

Fig. 3. The kinetics of inactivation of GTP cyclohydrolase I by oGTP. A plot of the pseudo first order rate constant (K'), was plotted as a function of the concentration of oGTP. The constant, K', was calculated from the slope of the lines in Fig. 2.

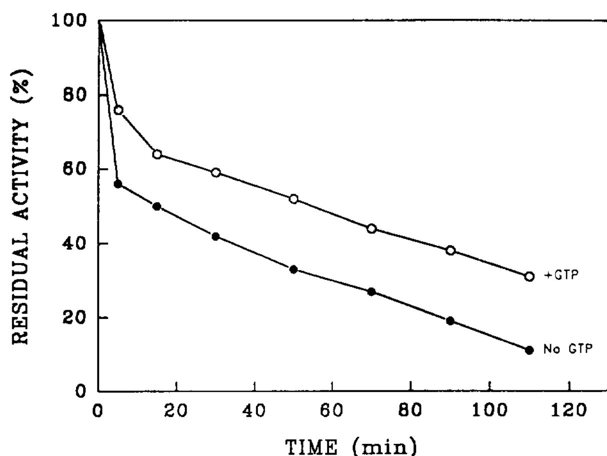

Fig. 4. Substrate protection against inactivation of GTP cyclohydrolase I by oGTP. 1.2 nmol of the enzyme was incubated in a final volume of 0.5 ml of 50 mM KH_2PO_4 (pH 7.0), 5 mM EDTA, which contained 1 mM oGTP and 10 mM GTP. Prior to incubation, the enzyme solution was preincubated at 30℃ for 10 min in the presence of 10 mM GTP, ●, and in the absence of GTP, ○.

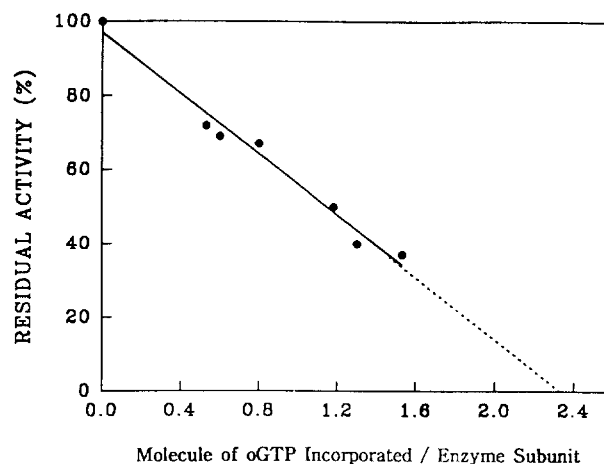

Fig. 5. Incorporation of radioactive oGTP per subunit of GTP cyclohydrolase I. The enzyme (0.57 nmol) was incubated with various concentrations (0.1~2.5 mM) of [8,5'-^{3}H]oGTP. The remaining enzyme activity and the radioactivity incorporated into the protein was determined as described in "Results".

corresponded to the enzyme peak. These results indicate the formation of a stable covalent bond between the enzyme and oGTP without reduction by borohydride.

Kinetics of inactivation

The enzyme was incubated with different concentrations of oGTP (0.01 mM, 0.25 mM, 0.5 mM, 1 mM, and 1.5 mM), and the activity was measured at given times. As shown in Fig. 2, inactivation was dependent upon the concentration of oGTP, and the process was biphasic with a rapid reaction occurring immediately upon exposure of the enzyme to oGTP, followed by a slow inactivation continuing for more than 100 min. The data presented in Fig. 3 show the relation between the concentration of oGTP and the constant, K', obtained from the slope of the lines presented in Fig. 2. The extent of inactivation is proportional to the low concentrations of oGTP and become saturated at high concentrations of oGTP. This inactivation exhibiting saturation kinetics suggests that oGTP can act as an affinity label for the enzyme. The K_i value for oGTP calculated from a double-reciprocal plot of the data in Fig. 3 was 0.25 mM.

To prove oGTP's role as an affinity label, it is necessary to demonstrate that the presence of the substrate should specifically protect the enzyme against the inactivation process. As shown in Fig. 4, the slow phase of inactivation was prevented by preincubation of the enzyme with 0.5 mM GTP, and the rapid phase was also inhibited significantly by the substrate.

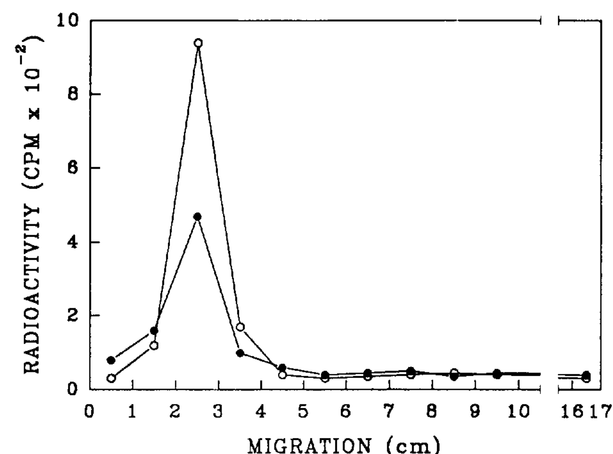

Fig. 6. Identification of the modified amino acid residue. [8,5'-^{3}H]oGTP-modified GTP cyclohydrolase I was hydrolyzed by a mixture of proteolytic enzymes and the material was subjected to ascending chromatography on Whatman 3 MM paper using 1-butanol/acetic acid/water(4/1/5, by volume) as the developing solvent. The paper was cut into 1 cm sections and the radioactivity of each section was measured (●-●). Lysyl-[8,5'-^{3}H]oGTP was used as the standard marker (○-○).

Stoichiometry of inactivation

The enzyme was incubated with various concentrations of [8,5'-^{3}H]oGTP for 20 min at 42℃. After incubation the enzyme activity was determined, and the remainder of the enzyme was precipitated using cold 5% trichloroacetic acid in the presence of 10 μg of bovine serum albumin, which acted as a carrier protein. The precipitate was collected by centrifugation, and washed three times in cold 5% TCA. The precipitated protein was then collected and radioactivity was counted. Extrapolation of the data shown in Fig. 5 reveals that

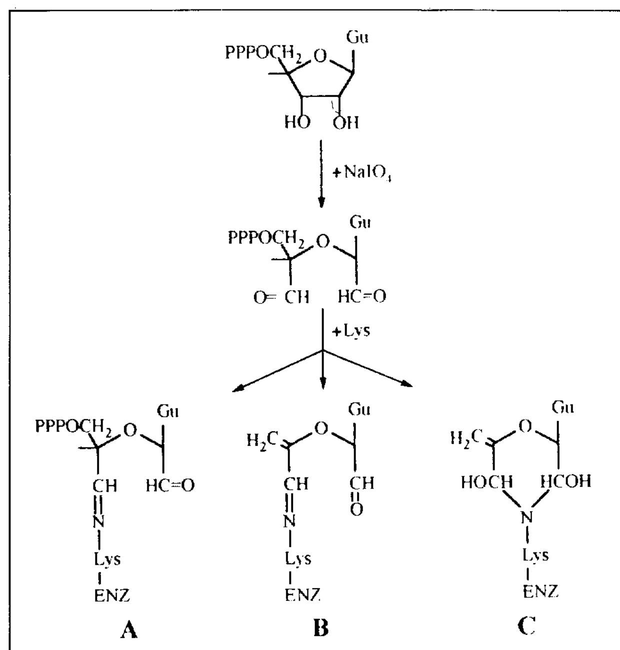

Fig. 7. Possible structures resulted from the reaction of active site lysine with oGTP. (A) Schiff base, (B) Conjugated Schiff base, (C) Morpholine derivative.

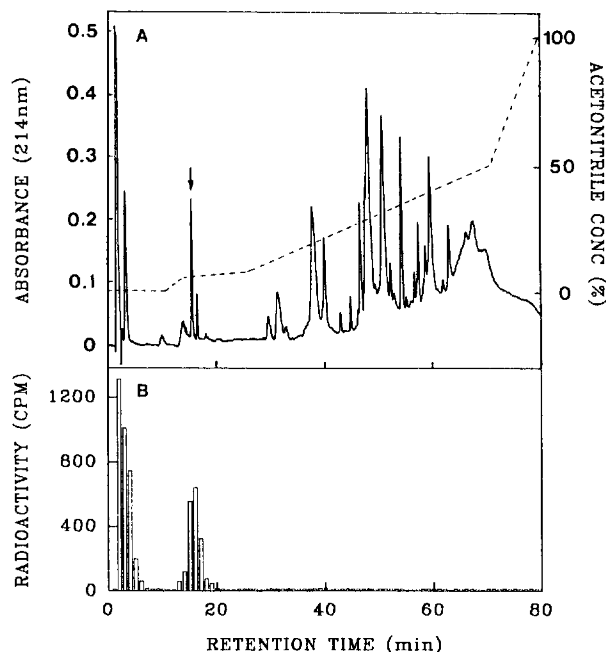

Fig. 8. Separation of ^{3}H-labeled peptide by HPLC. [8,5'-^{3}H] oGTP-labeled enzyme was cleaved with endoproteinase Lys-C, and the digest was subjected to reversed-phase HPLC on a C-18 Nova-pak column with a linear gradient of 0 to 50% acetonitrile (dashed line) in 0.1% trifluoroacetic acid. The elution profile (A) was monitored by absorbance at 214 nm (solid line) and the distribution of ^{3}H radioactivity was determined (B). The fraction containing the major radioactivity peak (indicated by a arrow) was used for amino acid sequencing.

2.3 molecules of [8,5'-^{3}H]oGTP were bound per enzyme subunit.

Identification of the modified amino acid

The participation of the lysine residue in the formation of covalent enzyme bonding with nucleotides of dialdehyde has been reported (Easterbrook-Smith *et al.*, 1976; Gregory and Kaiser, 1979; King and Carlson, 1981; Rabinkov and Amontov, 1990; White and Levy, 1987; Hountondji *et al.*, 1990). In the case of avian myeroblastosis virus reverse transcriptase, cystein is known to be involved in inactivation by oATP (Srivastava *et al.*, 1983).

To identify the residue modified by oGTP, GTP cyclohydrolase I was inactivated by [8,5'-^{3}H]oGTP, and the material was hydrolyzed with a combination of trypsin, chymotrypsin, and proteinase K. The digests were then developed on Whatman paper using lys-[8,5'-^{3}H] oGTP as a standard marker. The results shown in Fig. 6 indicate that a single radioactive compound obtained from the enzyme digest is identical to the standard lys-[8,5'-^{3}H]oGTP marker.

Reaction mechanism of oGTP with GTP cyclohydrolase I

Several structures are possible between oGTP and the lysine moiety of the enzyme. The simple Schiff base structure (Fig. 7A) is acid stable when reduced with borohydride (Dallocchio *et al.*, 1976). Two other possible forms are a conjugated Schiff base (Fig. 7B), and a morpholine derivative (Fig. 7C). The morpholine derivative is acid-labile, irrespective of borohydride reduction and, when treated with acid, the label is removed from the enzyme (Gregory and Kaiser, 1979).

To investigate the modified GTP cyclohydrolase I structure, the oGTP-inactivated enzymes, with and without borohydride treatment, were hydrolyzed in 6 N HCl for 24 h, and their amino acid compositions were analyzed. There was no difference in the number of lysine residue between the two cases. These results suggest that the modifier was removed from the modified enzyme by acid hydrolysis, and the structure formed between the active site lysine and oGTP is not a conjugated Schiff base but a morpholine derivative.

An additional experiment was performed to demonstrate elimination of the oGTP triphosphate group during modification of the enzyme by oGTP. When the enzyme was modified by [8,5'-^{3}H]oGTP and [α-^{32}P] oGTP respectively, ^{3}H incorporation corresponded to 2.3 mol, whereas the value of ^{32}P was less than 0.02 mols per subunit. The difference in these values suggests strongly an intramolecular rearrangement reaction accompanied by elimination of the triphosphate group of oGTP.

Table 1. Amino acid sequence of ^{3}H-labeled peptide[a]

Cycle No.	PTH-amino acid	Yield (pmol)	Predicted[b]
1	Pro	16	Pro
2	Ser	7	Ser
3	Leu	25	Leu
4	Ser	6	Ser
5	Lys	22	Lys
6	ND	ND	Glu
7	ND	ND	Ala
8	ND	ND	Ala

[a]The ^{3}H-labeled peptide was prepared as shown in Fig. 8. The peptide was subjected to amino acid sequencing as described in "Materials and Methods".
[b]Amino acid residues at amino-terminal position predicted from nucleotide sequence of the gene (Katzenmeier *et al.*, 1991). PTH: phenylthiohydantoin; ND.: not determined.

Isolation and sequencing of the ^{3}H-labeled peptide

The enzyme was affinity-labeled by incubation with [8,5′-^{3}H]oGTP, and cleaved using endoproteinase Lys-C. The digest was subjected to reversed-phase HPLC to purify the active site peptide. One ^{3}H-labeled peptide was isolated (Fig. 8) and its amino acid sequence was determined. The sequence started with Pro, followed by Ser-Leu-Ser-Lys (Table 1). No residues were identified after the 6th cycle. This result indicates that the peptide bond at the C-terminal side of the lysine residue was cleaved normally by the action of endoproteinase Lys-C, in spite of its modification. The lysine residue itself was detected by the amino acid sequencer. This indicates that the ^{3}H-label was removed from the lysine residue by the action of trifluoroacetic acid during Edman degradation.

The above sequence was compared with the primary structure of the enzyme deduced from the gene sequence (Katzenmeier *et al.*, 1991). The sequence corresponded to that of an amino-terminal.

Discussion

If an analog acts specifically as an affinity label for the active site of an enzyme, it should meet the following criteria (Colman, 1983; Wold, 1977; Phillips, 1977): 1) rate saturation effect; 2) protection against inactivation by the substrate; and 3) stoichiometric incorporation of the analog. Inactivation of GTP cyclohydrolase I by oGTP satisfied the above conditions, and this establishes that oGTP can act as an affinity label for the enzyme.

The biphasic process of inactivation is noticeable. A similar phenomenon was observed in the inactivation of 2-keto-3-deoxy-6-phospho gluconic aldolase from *Pseudomonas putida* (Moleche, 1967). Biphasic kinetics suggest the reversible formation of a complex between oGTP and the enzyme prior to covalent modification, as shown in the following equation:

$$E + oGTP \rightleftharpoons E\text{-}oGTP \longrightarrow E_{inact}$$

where E represents the free enzyme; E-oGTP represents the reversible enzyme-oGTP complex; and E_{inact}, represents the inactivated enzyme.

Three possible reactions can occur between a dialdehyde derivative of purine nucleotides and the amino group of lysine. A simple Schiff base structure was proposed for the interaction of several enzymes with the dialdehyde derivative of ATP (Easterbrook-Smith *et al.*, 1976; Dallochio *et al.*, 1976; Kotchetkov *et al.*, 1977; Maccioni *et al.*, 1979; Ranieri-Raggi and Raggi, 1976; Kumar *et al.*, 1979; Westcott *et al.*, 1980). The Schiff base can be subsequently reduced by sodium borohydride to yield a stable secondary amine (Easterbrook-Smith *et al.*, 1976; Fig. 7A). The reduced product is acid-stable (Dallocchio *et al.*, 1976).

In other cases the reaction between periodate-oxidized ATP and lysine does not involve the formation of a simple Schiff base (Gregory and Kaiser, 1979; King and Carlson, 1981; Lowe and Beechey, 1982; Miziorko *et al.*, 1990; Rabinkov and Amontov, 1990). The dialdehyde derivative of ATP can react reversibly with the enzyme to form a conjugated Schiff base. Elimination of the triphosphate group occurs, leaving an enzyme-bound product containing a conjugated Schiff base (Fig. 7B). The structure is known to be more stable than the simple Schiff base (Monsan *et al.*, 1975). The third possibility is the formation of a morpholine derivative. The dialdehyde derivative of ATP reacts with the amino group of the lysine residue to produce a dihydroxymorpholine derivative, then the triphosphate group is eliminated, as shown in Fig. 7C. This product is also stable without reduction by borohydride. The morpholine derivative is acid-labile and, when treated with acid, it is removed from modified enzymes (Gregory and Kaiser, 1979; King and Carlson, 1981).

The amino-terminal peptide Pro-Ser-Leu-Ser-Lys was labelled by the substrate analog. This result suggests that the active site of the enzyme may be located between subunits of the oligomeric protein because the amino-terminal residue is not usually buried inside of the tightly folded molecule (Lehninger, 1979). However, the lysine residue of this peptide may not possess a catalytic function. According to Kochetkov *et al.* (1977), the lysine residue linked to the dialdehyde derivative of the nucleotide is considered as a cationic site which anchors the polyphosphate group of the nu-

cleotide substrate to the enzyme.

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