A Chromophoric and Fluorescent Analog of GTP, 2′,3′-O-(2,4,6-Trinitrocyclohexadienylidene)-GTP, as a Spectroscopic Probe for the GTP Inhibitory Site of Liver Glutamate Dehydrogenase*

Received for publication, November 9, 1984

Toshiaki Hiratsuka

From the Department of Chemistry, Asahikawa Medical College, Asahikawa, Hokkaido 078-11, Japan

The ribose-modified chromophoric and fluorescent analog of ATP, 2′,3′-O-(2,4,6-trinitrocyclohexadienylidene)-ATP (TNP-ATP) (Hiratsuka, T., and Uchida, K. (1973) Biochim. Biophys. Acta 320, 635-647 and Hiratsuka, T. (1976) Biochim. Biophys. Acta 453, 293-297) has been widely used as an ATP analog for various ATPases. Although the corresponding analog of GTP, 2′,3′-O-(2,4,6-trinitrocyclohexadienylidene)-GTP (TNP-GTP) should be useful for the study of various GTP-requiring enzymes, it is difficult to prepare TNP-GTP by the conventional method.

In the present study, we succeeded in the synthesis of TNP-GTP with the use of an alternative method. The analogs of GDP, GMP, and guanyl-5′-y1 imidodiphosphate (Gpp(NH)p) were also synthesized. Visible absorption and fluorescent properties of TNP-GTP, TNP-GDP, TNP-GMP, and TNP-Gpp(NH)p were quite similar to those of TNP-ATP. TNP-GTP was found to be able to replace GTP as an inhibitor for bovine liver glutamate dehydrogenase. The enzyme was inhibited by TNP-GTP to a maximum extent of 54% at saturating concentrations of the analog with a Kᵢ of 2.7 µM. TNP-Gpp(NH)p and other ribose-modified fluorescent analogs of GTP, 3′-O-anthraniloyl-GTP and 3′-O-(N-methylanthraniloyl)-GTP (Hiratsuka, T. (1983) Biochim. Biophys. Acta 742, 496-508), also inhibited the enzymatic activity. Binding of TNP-GTP to the enzyme was characterized by a 5.6-fold enhancement in analog fluorescence. In the presence of NADH, the limiting fluorescence enhancement of the bound analog decreased to 2.7-fold. As determined by fluorometric titration, the maximum number of TNP-GTP binding sites on the enzyme was 1.9 mol/mol of subunit with a Kᵢ of 0.66 µM in the absence of NADH and 2.2 mol/mol of subunit with two Kᵢ values of 0.11 and 0.71 µM in the presence of NADH. These observations suggest that NADH binding increases the affinity of only 1 mol of the 2 mol of TNP-GTP bound to the enzyme.

These spectroscopic and biological properties of TNP-GTP should make this analog useful as a chromophoric and fluorescent probe for studies not only of glutamate dehydrogenase but also of various GTP-requiring enzymes, which have a high specificity for the base moiety of GTP.

Like ATP, GTP is critically involved in a number of complex and important biological processes. Additionally, a few enzymes, i.e. mammalian succinyl-CoA synthetase (1), phosphoenolpyruvate carboxykinase (2) and guanylate cyclase (3), are specific for GTP. One approach to study mechanisms of these biological reactions is the use of a reporter-labeled GTP, i.e. a chromophoric or fluorescent analog of GTP. Ribose-modified GTP analogs (4-6) are especially useful for GTP-requiring enzymes which are sensitive to alteration in the base or phosphoryl moiety of the nucleotide.

We have previously synthesized the ribose-modified chromophoric and fluorescent analog of ATP, TNP-ATP, by the reaction of ATP with TNBS (7,8). TNP-ATP has absorption maxima in the visible region (7,8), and fluoresces in the range of 530-560 nm (8,9). Furthermore, its visible absorption (7,8) and fluorescence emission (8,9)are sensitive to solvent polarity, making it an extremely good chromophoric and fluorescent probe. Because of these spectroscopic properties of this analog, TNP-ATP has been widely used as an ATP analog in the study of various ATP-requiring systems: myosin ATPase (7,9-11), (Na⁺ + K⁺)-ATPase (12), (Ca⁺₂ + Mg⁺₂)-ATPase (13,14), gastric (H⁺ + K⁺)-ATPase (15), mitochondrial ATPase (16,17), vacuolar membrane ATPase (18), chloroplast coupling factor 1-ATPase (19), myokinase (8), pyridoxal kinase (20), aspartokinase (21), eukaryotic protein chain initiation factor (22), and actin (23,24). These results encouraged us to synthesize the GTP analog corresponding to TNP-ATP. However, it is difficult to prepare the O-TNP-derivative of GTP (TNP-GTP, Fig. 1) by the conventional method (7,8) because the reaction of guanosine derivatives with TNBS yields the mixture of N-TNP, O-TNP, and N,O-bis-TNP derivatives (25).

In the present study, we succeeded in the synthesis of TNP-GTP, TNP-GDP, TNP-GMP, and TNP-Gpp(NH)p using TNCB instead of TNBS. Here we describe the synthesis of these analogs and their spectroscopic and biological properties. The studies reported here demonstrate that visible absorption and fluorescent properties of analogs are quite similar to those of TNP-ATP (8), and TNP-GTP can replace GTP as an allosteric inhibitor for bovine liver glutamate dehydrogenase. TNP-GTP, therefore, should be a suitable spectroscopic probe for the study of various GTP-requiring enzymes.

EXPERIMENTAL PROCEDURES

Materials

ATP, GMP, and NADH were purchased from Kanto Co. GDP and GTP were from Yamasa Shoyu Co. Gpp(NH)p was from Boehringer.

The abbreviations used are: TNP, 2′,3′-O-(2,4,6-trinitrocyclohexadienylidene) in complexes of ATP, GTP, GDP, GMP and Gpp(NH)p at neutral and basic pH including 2,4,6-trinitrophenyl in N-TNP derivatives; Gpp(NH)p, guanyl-5′-y1 imidodiphosphate; TNBS, 2,4,6-trinitrobenzenesulfonic acid; TNCB, 2,4,6-trinitrochlorobenzene; TLC, thin layer chromatography.
ringer Mannheim. TNP-ATP was prepared by the method described previously (7, 8). TNCB was from Tokyo Kasei Kogyo Co. and was used without further purification. Silica gel (Silica Gel 60) and cellulose (Avicel SP) TLC plates were from Merck and Funakoshi Chemical Co., respectively. Other reagents were of reagent or biochemical research grade.

Bovine liver glutamate dehydrogenase, purchased as a crystalline suspension in ammonium sulfate from Sigma, was dialyzed for 20 h at 4°C against three changes of 0.1 M potassium phosphate buffer (pH 7.0). The dialyzed material was centrifuged for 15 min at 18,000 rpm to remove insoluble material. The protein concentration was determined with the value E280 nm = 9.7 (27). The enzyme was used within 4 days. A molecular weight of 36,100 for identical peptide chains was used in the calculations (28).

**Methods**

---

**Enzyme Assays**—Glutamate dehydrogenase activity was assayed spectrophotometrically at 340 nm by measuring the oxidation of NADH. The total volume of the assay solution was 3.0 ml. When the activity was measured in the presence of GTP and the analog as inhibitors, the nucleotide concentrations used were 0.02-1.9 mM GTP or the analog required for half-maximum inhibition. The substrate concentrations used were 28-45% (Table I).

**Spectral Measurements**—Absorption spectra were measured at room temperature with a Shimadzu double-beam spectrophotometer, Model UV-200. Fluorescence emission spectra (uncorrected) were recorded at 25°C in a thermostated Hitachi fluorescence spectrophotometer, Model MFP-4, as described previously (8). Excitation wavelengths were 408 or 470 nm. The slit widths on the excitation and emission monochromators were 5 or 10 nm.

**TNP-GTP Binding Measured by Fluorescence Titration**—The binding of TNP-GTP to glutamate dehydrogenase was measured at 25°C in the presence and absence of 100 mM NADH in 10 mM Tris acetate (pH 8.0) containing 7 mM potassium phosphate and 10 mM EDTA. Samples were excited at 470 nm to avoid errors arising from absorption by NADH, and emission was measured at 548 and 545 nm in the presence and absence of the nucleotide concentration, respectively. Samples were allowed to incubate for 5 min prior to fluorescence measurements in order to attain equilibrium.

The dissociation constants and number of TNP-GTP binding sites on the enzyme were determined as follows. The fluorescence of TNP-GTP was measured in the presence, F, and absence, Fo, of enzyme (1.0 μM), and the ratio of fluorescence (F/Fo) was used to calculate [TNP-GTP]bound from

\[
[TNP\text{-}GTP]_{\text{bound}} = \frac{[TNP\text{-}GTP]_{\text{total}}}{Q-1} \cdot \frac{F}{F_0} - 1
\]  

where Q is the enhancement of TNP-GTP fluorescence for bound ligand. The enhancement factor, Q, was measured by titrating a fixed amount of TNP-GTP (2.7 μM) with increasing amounts of the enzyme in a concentration range of 0.5-4 μM. A double-reciprocal plot of total protein concentration versus observed fluorescence was extrapolated to infinite protein concentration in order to determine the value of Q. Enzyme concentration factors, Q, in the presence and absence of NADH were 2.7 and 5.6, respectively. The amount of free TNP-GTP is obtained from the difference of the total TNP-GTP and calculated bound TNP-GTP.

The data were analyzed in terms of the Scatchard equation:

\[
\frac{B}{[L]_E} = \frac{n}{K_d} - \frac{B}{K_d E_r} \tag{2}
\]

where [L] is the free TNP-GTP concentration, B and E are amounts of bound TNP-GTP and total enzyme, respectively, n is the number of binding sites, and Kd is the dissociation constant for the enzyme-TNP-GTP complex.

**Synthesis of GTP Analogs**—3'-O-(N-Methylanthraniloyl)-GTP and 3'-O-(N-4-Methylanthraniloyl)-GTP were prepared as described previously (4). 3'-O-(N-Methylanthraniloyl)-Gpp(NH)p was prepared by the procedure similar to that for 3'-O-(N-methylanthraniloyl)-GTP (4), except that Gpp(NH)p (0.16 mmol, lithium salt) was dissolved in 2 ml of water and allowed to react with N-methylisatoic anhydride (40 mg) by titrating with 1 N LiOH instead of NaOH. The reaction products were chromatographed on a column of Sephadex LH-20 (2.5 × 83 cm) eluted with water (4). Fractions of 2.5 ml were collected. 3'-O-(N-Methylanthraniloyl)-Gpp(NH)p was eluted after the peak of unreacted Gpp(NH)p. Peak fractions of the analog were pooled and evaporated to dryness at 25°C after neutralization (pH 6.8) with 1 N HCl. The residue was dissolved in 1 ml of water. An excess of cold acetone (15 ml) was added, and the resultant precipitate was collected and washed with ether (20 ml). The material was dried on P2O5 in vacuo overnight. The analog was chromatographically pure as indicated by a single fluorescent spot, and free from starting materials and degradation products. The Kd values in Solvent I on cellulose were 0.31 for 3'-O-(N-methylanthraniloyl)-Gpp(NH)p and 2.1 for Gpp(NH)p. Absorption and fluorescent properties of 3'-O-(N-methylanthraniloyl)-Gpp(NH)p were virtually identical to those of 3'-O-(N-methylanthraniloyl)-GTP (4). Concentrations of 3'-O-anthraniloyl and 3'-O-(N-methylanthraniloyl) analogs were determined by absorption at 352 nm (ε = 4500 M⁻¹cm⁻¹) and at 366 nm (ε = 5700 M⁻¹cm⁻¹), respectively (4).

**TNP-analogs of GTP, GDP, and GMP** were prepared as follows. The nucleotide (0.5 mmol, sodium salt) was dissolved in 3 ml of water. The pH was adjusted to 9.6 with 4 N LiOH. To this solution, with continuous stirring, a crystalline suspension of TNCB (1 mmol) was added. The pH was maintained at 9.6 by titration with LiOH for 6 h at 38°C. After completion of the reaction, the pH of the reaction mixture was adjusted to 7.0 with concentrated HCl. The reaction mixture was left for 15 min in ice. The resultant suspension was centrifuged at 18,000 rpm for 10 min. An excess of cold ethanol (20 ml) was added to the supernatant fluid. After cooling in ice for 10 min, the precipitate was collected by centrifugation, dissolved in an equal volume of water, and the solution was neutralized with 1 N HCl. The solution was then placed on a Sephadex LH-20 column (2.5 × 68 cm, packed in water) (7, 8). This column was developed with water at a flow rate of about 60 ml/h. Fractions of 5 ml were collected. A separation profile was obtained after assays by TLC on cellulose; portions of the extract were collected in 2.5 ml of water. Fractions of 2.5 ml were collected. A separation profile was obtained after assays by TLC on cellulose; portions of the extract were collected in 2.5 ml of water. Fractions of 2.5 ml were collected.
TNP-GTP Binding to Glutamate Dehydrogenase

Table I

| Compound      | RF in system | Yield | Formula (M, in parentheses)                  | Analysis (calculated) (found) |
|---------------|--------------|-------|---------------------------------------------|-------------------------------|
|               | A            | B     | C                                           | C H N                         |
| TNP-GMP       | 0.59         | 0.36  | 0.52                                        | %                             |
| TNP-GDP       | 0.48         | 0.21  | 0.48                                        | %                             |
| TNP-GTP       | 0.38         | 0.12  | 0.53                                        | %                             |
| TNP-Gpp(NH)p  | 0.47         | 0.07  | %                                           | %                             |
| GMP           | 0.14         |       | 0.28                                        | %                             |
| GDP           | 0.10         |       | 0.27                                        | %                             |
| GTP           | 0.09         |       | 0.31                                        | %                             |
| Gpp(NH)p      | 0.10         |       |                                             | %                             |
| Picric acid   | 0.98         | 0.84  | 0.99                                        | %                             |

FIG. 2. Graphic presentation of chromatographic purification of TNP-guanine nucleotides. Reaction products were applied on a 2.5 X 68-cm column of Sephadex LH-20 packed in water. The flow rate was 60 ml/h; 5-ml fractions were collected. The material shown at the left was eluted in the fractions indicated.

Fraction treatments, N-TNP-guanine derivatives remain intact (25).
1) By the action of 1 N KOH for 18 h at 37 °C, none of the analogs was degraded, suggesting O-TNP-guanosine derivatives; N-TNP-guanine derivatives are almost completely degraded on this treatment (25).
2) All analogs exhibit absorption maxima at 408 and 470 nm in a medium at pH 8.0 (Fig. 3). This is characteristic of O-TNP-nucleosides (25) and O-TNP-ATP (7); absorption spectra of N-TNP-guanine derivatives exhibit a maximum at 430 nm (25).
3) The molar absorption coefficients (ε) for TNP-GTP (26,500 M⁻¹.cm⁻¹ at 408 nm, and 18,300 M⁻¹.cm⁻¹ at 470 nm) are essentially identical with those of TNP-ATP (7, 8), which implies that the base moiety of TNP-GTP has little effect on the chromophore (Fig. 3). The spectrum also exhibits an absorption maximum at 252 nm (ε = 24,100 M⁻¹.cm⁻¹) and a distinct shoulder at 280 nm, which is characteristic of guanine derivatives. There was no significant difference in absorption spectra of TNP analogs of GMP, GDP, GTP, and Gpp(NH)p (not shown).

Fluorescent Properties of TNP-GTP—Upon excitation with light in the 410- or 470-nm regions, TNP-GTP fluoresced similarly to TNP-ATP (8, 9). The uncorrected fluorescence emission of TNP-GTP in aqueous buffer forms a single band with a maximum at 552 nm (Fig. 4). The potential usefulness of the analog as a fluorescent probe of hydrophobic microen-
TNP-GTP Binding to Glutamate Dehydrogenase

Fig. 4. Uncorrected fluorescence emission spectra of TNP-GTP (---) and TNP-ATP (---) in water/N,N-dimethylformamide mixtures. All samples (1 μM) contained 10 mM Tris-HCl (pH 8.0). The percentages of N,N-dimethylformamide (v/v) and the emission maximum are indicated on the curves. Excited at 408 nm.

Environments is indicated by the fact that the position of emission maximum and the fluorescence intensity of the analog vary significantly with solvent polarity. As shown in Fig. 4, the intensity of TNP-GTP increases 5- and 24-fold in going from water to 40% and to 80% N,N-dimethylformamide, respectively. At the same time, the emission maximum is shifted to blue by 8 and 20 nm, respectively. Quantum yields of TNP-GTP were identical with those of TNP-ATP within the experimental error (±5%) in 0–80% N,N-dimethylformamide. Although data are not shown, all TNP-guanine nucleotides, including TNP-Gpp(NH)p, had similar fluorescent properties.

Kosower (29) has introduced an empirical polarity scale, the Z value, based on transition energies of a pyridinium-iodine complex in various solvents. We have also observed a good correlation between the Z value and fluorescent properties of TNP-guanine nucleotides. Results obtained with TNP-GTP are shown in Fig. 5. As expected, large changes in the fluorescence intensity and the emission maximum were observed with changes in the ethanol composition of ethanol/water mixtures. A good correlation was found between the emission maximum, as well as the fluorescence intensity, and a polarity scale, the Z value. These results allow ready use of these TNP-guanine nucleotides, as well as TNP-adenine nucleotides (8, 9), as fluorescent probes for hydrophobic regions of proteins.

Effect of TNP-GTP on Catalytic Activity of Glutamate Dehydrogenase—GTP is the natural inhibitor of glutamate dehydrogenase, which is the strongest and the most specific for this enzyme (30, 31). Our first interest was to determine whether the structural features of TNP-GTP resemble GTP sufficiently to allow acceptance by glutamate dehydrogenase as an inhibitor. As shown in Fig. 6A, TNP-GTP inhibits the enzymatic activity to a maximum extent of 54% at saturating

Fig. 5. Plots of fluorescence intensity and emission maximum of TNP-GTP (1 μM) versus the empirical solvent polarity scale, Z, proposed by Kosower (29). The Z values of the solvent in ethanol/water mixtures were taken from Ref. 29. Excited at 408 nm.

Fig. 6. Inhibition of glutamate dehydrogenase by TNP-guanine nucleotide analogs (A) and GTP (B). The enzymatic activity was assayed at 25 °C in 10 mM Tris acetate (pH 8.0), 10 μM EDTA, 5 mM α-ketoglutarate, 50 mM ammonium chloride, and 100 μM NADH. A, ●, TNP-GTP; O, TNP-Gpp(NH)p. B, experiments were performed in the presence () and absence (○) of 8.3 μM TNP-GTP.
concentrations of the analog with an inhibition constant, \( K_i \), of 2.7 \( \mu M \). When the terminal P-O-P moiety of TNP-GTP was replaced by a P-NH-P group, there was decreased binding of the analog to the enzyme, as evidenced by a 3.1-fold increase in the \( K_i \) value (8.4 \( \mu M \)), while the maximum extent of inhibition scarcely changed (i.e., TNP-Gpp(NH)p).

To determine whether TNP-GTP and GTP compete kinetically for the enzyme, the enzymatic activity as a function of GTP concentration was measured in the presence and absence of a constant TNP-GTP concentration (Fig. 6B). In the absence of TNP-GTP, the enzyme was inhibited by GTP to a maximum extent of 92% at saturating concentrations of the nucleotide with a \( K_i \) of 0.07 \( \mu M \). In the presence of 8.3 \( \mu M \) TNP-GTP, a \( K_i \) for the enzyme-GTP complex was measured to be 0.25 \( \mu M \), a value 3.6-fold higher than the actual \( K_i \) of 0.07 \( \mu M \) measured in the absence of TNP-GTP. Saturating levels of GTP caused a 92% inhibition of the enzymatic activity, which is the same as that observed in the absence of TNP-GTP. These results suggest that TNP-GTP competes kinetically with GTP for the enzyme, and that TNP-GTP bound to the enzyme can be totally displaced by excess GTP.

Other ribose-modified analogs of GTP and Gpp(NH)p were also used to test the role of hydroxyl groups of the ribose moiety and the triphosphate moiety in GTP for inhibition of the enzymatic activity of glutamate dehydrogenase. These results are summarized in Table II. For comparative purposes, the results obtained with GTP, TNP-GTP, and TNP-Gpp(NH)p are also listed. 3'-O-Anthraniloyl-GTP, 3'-O-(N-methylanthraniloyl)-GTP, and 3'-O-(N-methylanthraniloyl)-Gpp(NH)p, in which only the 3'-hydroxyl group of the ribose moiety is modified (4), showed a greater extent of maximum inhibition than the corresponding TNP analog, in which both the 2'- and 3'-hydroxyl groups are modified (Fig. 1). Taking into account that 3'-O-anthraniloyl-GTP showed an inhibitory effect of 90% of that of GTP at saturating levels of the analog, the 2'-hydroxyl group, rather than the 3'-hydroxyl one in GTP seems to be required for proper positioning of the GTP molecule to inhibit the enzyme completely. On the other hand, replacement of the P-O-P moiety of the GTP analog with the P-NH-P group resulted in little or no change in the maximum extent of inhibition, but there was a 2.5-3-fold increase in the \( K_i \) value (i.e., TNP-Gpp(NH)p and 3'-O-(N-methylanthraniloyl)-Gpp(NH)p).

The binding of TNP-GTP to glutamate dehydrogenase was reflected in the emission spectrum of the analog. The addition of 2.4 \( \mu M \) enzyme to a 2.7 \( \mu M \) TNP-GTP solution resulted in a 4.3-fold enhancement in the fluorescence (b in Fig. 7). Under these conditions of measurement, a blue shift in the emission maximum from 552 to 545 nm was observed. The enhancement factor of the bound analog extrapolated to infinite protein concentration was found to be 5.6. This fluorescence enhancement was reversed or prevented by addition of 5 mm GTP or 250 mm ammonium chloride, which competes kinetically with GTP (32). This observation suggests that TNP-GTP binds to the GTP inhibitory site of the enzyme.

In the presence of NADH, the fluorescence of a solution of TNP-GTP and enzyme was less enhanced. When 100 \( \mu M \) NADH was added to a 2.7 \( \mu M \) TNP-GTP solution containing 2.4 \( \mu M \) enzyme, the enhancement decreased from 4.3- to 2.3-fold (d in Fig. 7). At the same time, only a small red shift in the emission maximum from 545 to 548 nm was observed. The limiting enhancement of the bound analog in the presence of NADH was measured to be 2.7. On the other hand, the addition of NADH to a free TNP-GTP solution resulted in no change in the fluorescence (c in Fig. 7). These results suggest that, in the NADH-TNP-GTP-enzyme complex, the TNP moiety of the analog is either less buried or isless rigidly positioned than in the TNP-GTP-enzyme complex.

Fig. 7 (inset) shows a typical TNP-GTP fluorescence titration in the presence and absence of enzyme. In the presence of 1 \( \mu M \) enzyme, a large fluorescence enhancement was observed as described above. This enhancement appears to saturate since the slope of the plot of fluorescence versus TNP-GTP concentration in the presence of enzyme approaches that in the absence of enzyme. The concentration of bound TNP-GTP is calculated from Equation 1 and used to determine \( K_i \) in Equation 2. The dissociation constants for enzyme-TNP-GTP complexes and number of binding sites in the absence and presence of NADH were calculated by Scatchard analysis from Equation 2.

In the absence of NADH, glutamate dehydrogenase exhibits a linear Scatchard plot (Fig. 8A). The data yield a value of 1.9 for the number of TNP-GTP sites/mol of enzyme subunit, with a dissociation constant, \( K_d \), of 0.66 \( \mu M \). In contrast, a nonlinear Scatchard plot is observed in the presence of NADH.

### Table II

| Compound               | \( K_i \) (\( \mu M \)) | Maximal inhibition % |
|------------------------|-------------------------|----------------------|
| GTP                    | 0.07                    | 100                  |
| TNP-GTP                | 2.7                     | 89                   |
| TNP-Gpp(NH)p           | 8.4                     | 61                   |
| 3'-O-Anthraniloyl-GTP  | 3.6                     | 90                   |
| 3'-O-(N-Methylanthraniloyl)-GTP | 6.8 | 72                   |
| 3'-O-(N-Methylanthraniloyl)-Gpp(NH)p | 17  | 73                   |

* \( K_i \) represents the concentration of GTP or the analog required for half-maximum inhibition.

* The maximum extent of inhibition at saturating concentrations of GTP was taken as 100%.
TNP-GTP Binding to Glutamate Dehydrogenase

**FIG. 8.** Scatchard plots of TNP-GTP binding to glutamate dehydrogenase in the presence and absence of NADH. TNP-GTP binding was measured by fluorescence titration as described under “Experimental Procedures.” A, enzyme (1 μM) in the absence of NADH. B, enzyme (1 μM) in the presence of 100 μM NADH. Buffer and other conditions are as described in Fig. 7.

(8B). Despite the nonlinearity of the plot, a value of 2.2 for the total number of TNP-GTP binding sites/mol of subunit is extrapolated on the abscissa. Precisely, the extrapolation yields 1.2 TNP-GTP binding sites/mol of subunit with a $K_D$ of 0.11 μM and 1.0 binding site with a $K_D$ of 0.71 μM, which is similar to the value obtained in the absence of NADH (0.66 μM). These results suggest that NADH enhances the binding of only 1 of the 2 mol of TNP-GTP bound to the enzyme.

**DISCUSSION**

Okuyama and Satake (33) have reported that the TNP group of TNCB is introduced into hydroxyl groups of Tyr, Ser, Thr, and alcohols more rapidly than that of TNBS. This is also the case with the modification of guanosine derivatives. The use of TNCB instead of TNBS is very effective for the synthesis of O-TNP-guanine nucleotides (Fig. 1) as revealed in the present paper. In contrast, the reaction of guanosine derivatives with TNBS yields the mixture of N-TNP, O-TNP, and N,O-bis-TNP derivatives (25). These results suggest that TNCB is more reactive than TNBS toward hydroxyl groups of the ribose moiety of guanosine derivatives.

TNP-ATP, in which adenine replaces the guanine in TNP-GTP, has been previously synthesized (7, 8). To be used as an “environmental probe” for proteins, the molecule must be highly sensitive to some indicator of local environment, e.g., polarity and viscosity. Previous studies (7–9) clearly indicated that the TNP group in TNP-adenine nucleotides is very useful not only as a chromophoric but also as a fluorescent probe for proteins. Because of these spectroscopic properties of the TNP group, TNP-ATP has been widely used in the study of various ATP-requiring systems (7, 9–24). Especially, TNP-adenine nucleotides are useful for the study of nucleotide binding because fluorescence of the analog is greatly enhanced upon binding to proteins: 11-fold for (Na$^+$ + K$^+$)-ATPase (12), 9–16-fold for aspartokinase (21), 7-fold for mitochondrial ATPase (16) and (Ca$^{2+}$ + Mg$^{2+}$)-ATPase (13), and 4–5-fold for glutamate dehydrogenase (34, 35). The present study revealed that spectroscopic properties of TNP-GTP are quite similar to those of TNP-ATP, and binding of TNP-GTP to glutamate dehydrogenase results in a 2.7- and 5.6-fold fluorescence enhancement in the presence and absence of NADH, respectively. TNP-GTP, therefore, should be a suitable spectroscopic probe for the study not only of glutamate dehydrogenase but also of various GTP-requiring enzymes.

Bovine liver glutamate dehydrogenase is an allosteric enzyme composed of six identical subunits. The enzyme contains several nucleotide sites/subunit, including sites for the activator ADP and the inhibitor GTP (30, 31). Although the average distances between the ADP and GTP sites, and the ADP or GTP and catalytic sites have been recently measured by fluorescence energy transfer (34, 35), the number of GTP binding sites on the enzyme has been very controversial. Frieden and Colman (36) and Dieter et al. (37) have indicated the existence of one GTP binding site/subunit. In contrast, Bell et al. (38) have recently reported the existence of two GTP binding sites. Pal and Colman (39) have also demonstrated that the second GTP site is demasked by NADH binding. Our present study revealed the binding of 2 mol of TNP-GTP/mol of enzyme subunit. The relationship of TNP-GTP to the GTP inhibitory site on the enzyme has been established under “Results.” The enzymatic activity was inhibited to a maximum extent of 54% by TNP-GTP, and through kinetic measurements, competition with the natural inhibitor, GTP, was demonstrated.

NADH enhances the binding of only 1 of the 2 mol of TNP-GTP bound to glutamate dehydrogenase. Pal and Colman (39) have also reported a similar result in the binding study of GTP in the presence of NADH although, unlike TNP-GTP, GTP is accessible to only one of the two sites in the absence of coenzyme. Therefore, there are high and low affinity sites for GTP or TNP-GTP on the enzyme in the presence of NADH. However, affinity of TNP-GTP for both sites appears to be higher than GTP as judged from the dissociation constants: 0.61 and 18 μM for GTP (39), which were measured at the lowest enzyme concentration level used (0.2 mg/ml), and 0.11 and 0.71 μM for TNP-GTP. This is also the case with TNP-adenine nucleotides. Several investigators have previously reported the higher affinity of TNP-adenine nucleotide over the corresponding natural nucleotide for various ATPases (7, 8, 12, 13, 16) and glutamate dehydrogenase (34). Thus, increased affinity of TNP-GTP may result from electrostatic interaction of the negative charge on the TNP moiety (Fig. 1) with a cationic amino acid residue at or near the location of ribose in the GTP inhibitory site.

Our results also revealed that fluorescence of TNP-GTP is less enhanced by the enzyme in the presence of NADH than in the absence of the coenzyme. This suggests that the microenvironment of the TNP moiety becomes less hydrophobic or less rigid upon binding of the coenzyme to the TNP-GTP-enzyme complex. On the basis of the maximal extent of fluorescence enhancement and the data shown in Fig. 5, the hydrophobicity of microenvironment around the TNP moiety may be considered as identical to 40 and 24% ethanol for the TNP-GTP-enzyme and NADH-TNP-GTP-enzyme complexes, respectively.

In conclusion, TNP-guanine nucleotides, as well as TNP-adenine nucleotides (7–9), are useful as chromophoric and fluorescent probes for hydrophobic regions in proteins. Especially, TNP-GTP is expected to have a wide applicability in probing the nucleotide sites of GTP-requiring enzymes. Alternatively, TNP-GTP may have a wide application in fluorescence energy transfer studies to investigate the structure of proteins. Although relatively low quantum yields of the analog (8) preclude its use as an energy transfer donor, this is an excellent acceptor for blue-emitting fluorophores, such as 3'-O-anthraniloyl and 3'-O-(N-methylanthraniloyl) derivatives of nucleotides, including ATP and GTP (4–6),

troscopic probe for the study not only of glutamate dehydrogenase but also of various GTP-requiring enzymes.
cAMP and cGMP (40), and N-arylamino-naphthalene sulfo-

Acknowledgments—I am indebted to Professor K. Uchida for his encouragement and support. I am grateful to K. Nakamura for typing the manuscript.

REFERENCES

1. Sanadi, D. R., Gibson, D. M., Ayengar, P., and Jacob, M. (1956) J. Biol. Chem. 218, 505-520
2. Chang, H. C., Maruyama, H., Miller, R. S., and Lane, M. D. (1966) J. Biol. Chem. 241, 2421-2430
3. Macchia, V., Varrone, S., Weissbach, H., Miller, D. L., and Pastan, I. (1975) J. Biol. Chem. 250, 6214-6217
4. Hiratsuka, T. (1983) Biochim. Biophys. Acta 742, 496-508
5. Hiratsuka, T. (1984) J. Biochem. (Tokyo) 96, 147-154
6. Hiratsuka, T. (1984) J. Biochem. (Tokyo) 96, 155-162
7. Hiratsuka, T., and Uchida, K. (1973) Biochim. Biophys. Acta 320, 635-647
8. Hiratsuka, T. (1982) Biochim. Biophys. Acta 719, 509-517
9. Hiratsuka, T. (1976) Biochim. Biophys. Acta 453, 293-297
10. Moss, D. J., and Trentham, D. R. (1983) Biochemistry 22, 5261-5268
11. Moczydlowski, E. G., and Fortes, P. A. G. (1981) J. Biol. Chem. 256, 780-784
12. Dupont, Y., Chapron, Y., and Pougeois, R. (1982) Biochem. Biophys. Res. Commun. 106, 1272-1279
13. Watanabe, T., and Inesi, G. (1982) J. Biol. Chem. 257, 11510-11516
14. Faller, L. D., Rabon, E., and Sachs, G. (1983) Biochemistry 22, 4676-4685
15. Grubmeyer, C., and Penefsky, H. S. (1981) J. Biol. Chem. 256, 3718-3727
16. Schäfer, G. (1982) FEBS Lett. 139, 271-275
17. Bowman, E. J. (1983) J. Biol. Chem. 258, 15238-15244
18. Bruist, M. F., and Hammes, G. G. (1982) Biochemistry 21, 3370-3377
19. Churchich, J. E., and Wu, C. (1981) J. Biol. Chem. 256, 780-784
20. Broglie, K. E., and Takahashi, M. (1983) J. Biol. Chem. 258, 12940-12946
21. Seel, S. N., Schmidt, A., and Marcus, A. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 6562-6565
22. Dos Remedios, C. G., and Cooke, R. (1984) Biochim. Biophys. Acta 788, 193-205
23. Tao, T., Lamkin, M., and Lehrer, S. S. (1983) Biochemistry 22, 3059-3066
24. Azezami, M., and Iwai, K. (1964) J. Biochem. (Tokyo) 55, 346-348
25. Ah-Kow, G., Terrier, F., Pouet, M-J., and Simonnin, M-P. (1980) J. Org. Chem. 45, 4399-4404
26. Olson, J. A., and Anfinsen, C. B. (1952) J. Biol. Chem. 197, 67-79
27. Smith, E. L., Landon, M., Fiszkiewicz, D., Brattin, W. J., Langley, J. J., and Malamed, M. D. (1970) Proc. Natl. Acad. Sci. U. S. A. 67, 724-730
28. Kosower, E. M. (1968) J. Am. Chem. Soc. 80, 3253-3260
29. Frieden, C. (1963) J. Biol. Chem. 238, 3286-3299
30. Goldin, B. R., and Frieden, C. (1972) Curr. Top. Cell. Regul. 4, 77-117
31. Cross, D. G., and Fisher, H. F. (1970) J. Biol. Chem. 245, 2612-2621
32. Okuyama, T., and Satake, K. (1960) J. Biochem. (Tokyo) 47, 454-466
33. Jacobson, M. A., and Colman, R. F. (1983) Biochemistry 22, 4247-4257
34. Jacobson, M. A., and Colman, R. F. (1984) Biochemistry 23, 3789-3799
35. Frieden, C., and Colman, R. F. (1967) J. Biol. Chem. 242, 1705-1715
36. Dieter, H., Koberstein, R., and Sund, H. (1981) Eur. J. Biochem. 115, 217-226
37. Bell, J. E., Smith, T. J., and Bell, E. T. (1984) Fed. Proc. 43, 1817
38. Pal, P. K., and Colman, R. F. (1979) Biochemistry 18, 838-845
39. Hiratsuka, T. (1982) J. Biol. Chem. 257, 13354-13358
40. Turner, D. C., and Brand, L. (1968) Biochemistry 7, 3381-3390