Synthesis and Utilization of 8-Azidoguanosine 3'-Phosphate 5'-[5'-32P]Phosphate

PHOTOAFFINITY STUDIES ON CYTOSOLIC PROTEINS OF ESCHERICHIA COLI*

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A family of guanosine 3',5'-phosphorylated nucleotides have been postulated to have pleiotypic regulatory properties in prokaryotes during the stringent response. To study proteins which may interact with nucleotides of this homologous series, a photoactive analog of guanosine 3',5'-diphosphate has been synthesized. The analog, 8-azidoguanosine 3'-phosphate 5'-[5'-32P]phosphate, proved to be an effective photoaffinity probe for two nucleotide-binding proteins of Escherichia coli sonicates. It predominately photolabels two proteins with approximate molecular weights of 86,000 and 65,000 (p86 and p65, respectively). The K_d for p65 was approximately 10 μM; that for p86 was not determined. The nucleotide-binding sites were characterized by photolabeling in the presence of various nucleotides. The nucleotides guanosine 3',5'-diphosphate, guanosine 3'-monophosphate 5'-diphosphate, and GMP appeared only slightly less effective. The relative lack of effectiveness of guanosine 3'-diphosphate 5'-monophosphate inhibiting photolabeling of either protein supports observations that this nucleotide does not have a regulatory role in E. coli. The results presented indicate that the 8-azidoguanosine analogs of this homologous series will prove to be effective probes for studying the protein-nucleotide interactions involved in the stringent response.

Many microorganisms respond to nutritional deprivation by the synthesis of "magic-spot" compounds, i.e. ppGpp1 and ppGpp (1). During this stringent response, the tetraprophosphate regulates cellular metabolism such that growth processes are inhibited but adaptive responses are encouraged. For example, stable RNA synthesis is inhibited, but some transcription is enhanced; protease activity is increased to provide amino acids for synthesis of new (adaptive) enzymes; and metabolic activities, in general, are slowed down while this process of adaptation is occurring (2). Since the discovery of these guanosine polyphosphates in 1969 (3), many mechanistic details have been elucidated, but many questions still remain unanswered.

A nucleotide photoaffinity probe may be defined as a nucleotide derivative which has affinities for binding sites and biological activity comparable to the unaltered nucleotide. Exposure to certain wavelengths of light converts the analog to a very reactive intermediate, typically a nitrene or carbene, which may result in covalent incorporation into the binding site if it is bound to a protein. There are certain advantages to using photoprobes over conventional chemical probes. One advantage is that K_d, K_a, and K, values can be determined in the absence of activating light. Another advantage is that complex systems such as ribosomes, membranes, or even whole-cell sonicates can be studied. In this way, the in vivo situation may be more closely approximated and information obtained that might be lost in a purified system. Many nucleotide photoaffinity probes have been synthesized and used successfully. The photoprobes [32P]8-N3cAMP and [γ-32P]8-N3ATP have been employed to study the mechanisms of action of cAMP-dependent protein kinase (4-6), and [32P] 8-N3cGMP has been used to study cGMP-dependent protein kinase (7). Photoactive analogs of GTP, i.e. [γ-32P]8-N3GTP and [γ,γ-32P]8-N3GTP, were used to study tubulin polymerization (8). Also, these nucleotide photoprobes may be successfully applied to many other biochemical problems. Considering the ubiquity and great importance of nucleotides to cellular metabolism and the general applicability of the photoaffinity approach, this technique will be more frequently utilized in the future.

Of the classical magic-spot compounds, only MSI, ppGpp, is known to be biologically active; MSII, ppGGpp, appears only to serve as precursor of ppGpp. Recently, a third magic-spot compound, ppGp (MSIII), was discovered in Escherichia coli. This nucleotide has been proposed to regulate transcription (9-11). Under certain conditions, other members of this guanosine nucleotide family may be found in vivo: ppGp and ppGp appeared in E. coli mutants with a defective (ppGpp degrading enzyme (12). It is entirely possible that these nucleotides could occur in wild-type bacteria if the hydrodase were inhibited. To properly study magic-spot protein interactions, it will be necessary to synthesize a complete series of 8-azidoguanosine nucleotides, starting with [5'-32P]p8N3Gp, including the two triphosphates, and finally pp8NGpp. The
present study involves synthesis and use of the first probe of this homologous series, 8-azidoguanosine 3'-phosphate 5'-[5'-32P]phosphate, to examine the potential usefulness of these analogs to detect and study proteins that interact with the magic-spot class of nucleotides.

MATERIALS AND METHODS

Guanosine nucleotides were obtained from P-L Biochemicals. DEAE-cellulose was purchased from Pharmacia. Polyethyleneimine TLC plates (PEI-cellulose F, 0.1-mm precoated plastic sheets) were manufactured by EM. Wild-type E. coli K12 (ATCC 10798) were obtained from the American Type Culture Collection. RNase A (from bovine pancreas, type IA, 71 units/mg) was purchased from Sigma; DNase I (2129 units/mg) from Worthington. Spectrotralyt dialysis tubing with a cutoff of 3500 daltons was used.

Preparation of E. coli Sonicates—The bacteria were grown at 37 °C to an A600 = 0.9 in 1 liter of nutrient medium, pH 7.2, containing 3 g of yeast extract (Difco), 8 g of nutrient broth (Difco), 10 g of NaCl, and 246 mg of MgSO4/7H2O. The cells were centrifuged at 14,727 × g for 10 min in a Sorvall RC58 centrifuge using the GSA head. The pellets were resuspended in 5 ml of 30 mM Tris/HCl, pH 8, 20% (w/v) sucrose. After addition of lysozyme to 100 µg/ml and EDTA to 10 mM, the cells were incubated 30 min on ice. The cells were sonicated for 2 min on ice at 100 watts (Bransonic 1510 with microprobe) after addition of MgCl2 to 20 mM. Following sonication, DNase I and RNase A were added to 5 µg/ml each. The sonicate was then dialyzed against 1 liter of 10 mM Tris, pH 7.4, 10% sucrose, 5 mM EDTA, 10 mM MgCl2, 120 mM KCl, 20 mM NaCl at 4 °C for 5.5 h. The resulting dialyzed sonicate was stored as 1-ml aliquots at −20 °C. The protein concentration of this sonicate was determined to be 54 mg/ml by the Bio-Rad procedure.

Photolabeling Experiments—Samples in the wells of an immunosport plate in an ice bath were photolyzed for 5 min at a distance of 10 cm in a Chromatovue Model CC20 light box (Ultra-Violet Products, Inc.). The wavelength of the light is 254 nm and the intensity is 160 microwatts/cm. The 50-µL samples contain 2 µl of E. coli sonicate (108 µg of protein) and various concentrations of [5'-32P]p8N3Gp and competing nucleotides (e.g., ATP, GTP, pppGpp, ppGp, pGp, and GMP) in 10 mM Tris, pH 7.4, 5 mM EDTA, 10 mM MgCl2, 120 mM KCl, 20 mM NaCl. Photolabeled samples were solubilized with 25 µl of 25% sucrose, 2.5% sodium dodecyl sulfate, 2.5 mg/100 ml pyronin Y, 25 mM Tris/HCl, pH 8.0, 25 mM EDTA, and 15.4 mg/ml DTT. The entire 754 sample was loaded onto the gel; in addition, the wells of the spot plate were washed with a 1:3 dilution of the solubilizing solution, and this wash was added to the gel as well. The Laemmli-type polyacrylamide gel consisted of a 4% stacking gel and a 7-14% running gel and was run for about 4 h at a 32-ma constant current, as previously reported (4).

Preparation of [γ-32P]ATP—[γ-32P]ATP was synthesized by the Glynn-Chappell procedure (14) with some modifications (15). After 20 min of incubation at room temperature, the reaction mixture was added to a DEAE-cellulose column (1.6 × 24 cm) and the [γ-32P]ATP eluted with a 300-ml 0-0.6 M triethylammonium bicarbonate gradient. The major radioactive peak following the minor [32P]Pr peak was pooled, rotary-evaporated, and identified as [γ-32P]ATP. After several additions of methanol to the residue followed by evaporation (to remove triethylamine), the [γ-32P]ATP was redissolved in a small volume of methanol. Specific activity was determined by UV spectrophotometry and liquid scintillation counting. The reaction was monitored, and the radioactive purity of the ATP was demonstrated with PEI TLC plates developed with 0.38 M KH2PO4, pH 3.4.

RESULTS

Synthesis of [5'-32P]p8N3Gp—The analog was prepared using polynucleotide kinase (P-L Biochemicals) with N8Gp and [γ-32P]ATP as substrates. 8N8Gp was prepared following the procedure published for 8-N3GMP except that the bromination buffer was 1 M phosphate, pH 3.4 (13) (see Fig. 1). The enzymatic conversion of 8N8Gp to 5'-32P]p8N3Gp was based on the reports of Lillehaug and Kleppe (16, 17). To a plastic tube was added 1 µmol of 8N8Gp and 0.3 µmol of [γ-32P]ATP in methanol. This solution was evaporated and the residue redisolved in 250 µl of 12 mM MgCl2, 125 mM KCl, and 84 mM Tris, pH 8.9. Ten units of polynucleotide kinase (2 µl) were added, and the reaction mixture was incubated for 2 h at 37 °C. To remove unreacted [γ-32P]ATP, glucose was added to 0.1 M concentration to 0.5 mg of hexokinase (Sigma type III, from yeast; 15 units/mg), and the solution was incubated 15 min at 25 °C. The solution was then loaded onto a column (2 × 37 cm) of DEAE-cellulose (previously equilibrated with triethylammonium bicarbonate), and the analog eluted with 400 ml of 0-0.5 M triethylammonium bicarbonate. The (radioactive) column elution profile is shown in Fig. 2 and consists of six peaks. (The elution can be followed with a UV monitor; however, UV light destroys the azide and even small losses of nanomolar quantities of analog are significant).

Identification of the components in each peak was done by comparing: (a) UV spectra, before and after photolysis; (b) UV spectra before and after treatment with DTT; (c) reactive-
polynucleotide kinase reaction, i.e., this enzyme is only known by the fact that 3' nucleotidase converted it to 8-N3GMP.

The late elution from the DEAE column also indicates it is the most negatively charged (most highly phosphorylated) species present.

Peak 6 contained one component of over 95% radiochemical purity. It was identified as p8N3GMP, by its UV spectrum and photosensitivity and by the fact that 3' nucleotidase converted it to 8-N3GMP. This identification is also supported by the selectivity of the polynucleotide kinase reaction, i.e., this enzyme is only known to add one phosphate. The late elution from the DEAE column also indicates it is the most negatively charged (most highly phosphorylated) species present.

Peak 6 was pooled and evaporated to dryness at 20 °C, dissolved in anhydrous methanol, evaporated to dryness three times (to remove excess triethylammonium bicarbonate), and extracted and stored at -20 °C in anhydrous methanol. [5'-32P]p8N3Gp appears to be much more sensitive to reduction and/or thermal breakdown than the corresponding 8-N3GMP analog. After the above isolation of this analog, with less than 5% detectable radionuclide impurities in the elution peak, there appeared two new contaminating substances of variable quantity with RF values of 0.31 and 0.24. These compounds could be produced from [5'-32P]p8N3Gp by reduction with DTT (RF = 0.31) or photolysis (RF = 0.24) as shown in Table I. Analog isolated by this procedure is over 80% radiochemically pure; the major contaminants are produced during the evaporation of the combined fractions in triethylammonium bicarbonate buffer. The analog could be further purified to over 95% radiochemical purity using a Waters high-pressure liquid chromatography system consisting of two Model 600A pumps and a model 660 solvent programmer. The column, packed with polyethyleneimine (2.5 x 20 mm, J. T. Baker Chemical Co.), was eluted with a linear gradient of 25-250 mM sodium phosphate, pH 7.5, at a flow rate of 0.5 ml/min. The reduced analog, p8NH2Gp, elutes first at 44 min, followed by p8N3Gp elution at 46 min. If present, p8BrGp elutes at 55 min. Purity of the p8N3Gp is supported by the expected λmax at 278 nm and an isobestic point at 243 nm (Fig. 4). Both the λmax and the isobestic point would be affected if significant contamination by either of the corresponding 8-bromo or 8-NH2 analogs was present. Both of these analogs have λmax values in this UV region (Fig. 1 and Table I) and would be the most likely compounds to cochromatograph with p8NH2Gp.

Digestion of [5'-32P]p8N3Gp with 3' nucleotidase confirmed the assigned structure of the analog since this specific enzyme produced [32P]8-N3GMP (Table I). However, after prolonged digestion, varying small amounts of some refractory compounds remained with RF values (TLC system B, Table I) of 0.33 and 0.25. DTT converted the faster-migrating compound (RF = 0.33) to the slower-moving compound (RF = 0.25). Using techniques previously described and a two-dimensional TLC system utilizing cellulose plates (developed in the first dimension with isobutyric acid/concentrated NH4OH/H2O, 66:1.7:33, and in the second dimension with saturated (NH4)2SO4/8.2% sodium acetate/isopropyl alcohol, 80:18:2) reported elsewhere (9), the refractory compounds were identified as [5'-32P]5'-p8N2HG2'p (RF = 0.33) and [5'-32P]5'-p8N3G2'p (RF = 0.25). Since polynucleotide kinase does not accept nucleotide 2'-phosphates as substrates (16), the 2' isomer must have been produced from the [5'-32P] p8N3Gp by incubation in the alkaline reaction mixture. The presence of this analog did not appear to affect the photo- labeling experiments. This is probably due to their low concentration, and the observation that the 2' isomer of p8Gpp is reportedly not synthesized in vivo (18) and is not known to be biologically active.

Photolabeling with [5'-32P]p8N3Gp—Fig. 5 shows an autoradiograph of a gel containing [5'-32P]p8N3Gp-photolabeled E. coli sonicates. Only two major bands with apparent molecular weights of 86,000 and 65,000 (referred to as p86 and p65, respectively) are visible after photolysis at 5 μM analog concentration (slot 2). Even at 30 μM [5'-32P] p8N3Gp, these are still the major bands; however, several minor bands appear at this higher concentration (slot 7). Slot 1 contains the Coomassie Blue-stained protein profile from which the autoradiograph was made. The Coomassie Blue bands which coincide with the bands on the autoradiograph are marked: p86 appears to be one of the visible Coomassie bands.
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### Table 1
Characteristics of radioactive products from the synthesis and purification of [5'-32P]p8N3Gp

| Peak | Treatment | \( R_F \) | \( \lambda_{max} \) | Identity |
|------|-----------|-----------|----------------|----------|
| 1    | None      | 0.67      | >230           | [32P]8N3Gp-6-P |
|      | +hv       | NC        | NC             | [32P]8N3Gp-G-6-P |
|      | +hv       | NC        | NC             | [32P]8N3Gp-G-6-P |
|      | +hv       | NC        | NC             | [32P]8N3Gp-G-6-P |
|      | DTT       | NC        | NC             | Photolysis products |
|      | 3'-Nucleotidase | NC | NC | [32P]8-N3GMP |
|      | 3'-Nucleotidase | NC | NC | [32P]8-N3GMP |
| 2    | None      | 0.54      | 278            | [32P]8-N3GMP |
|      | +hv       | (0.42)    | <240           | Photolysis products |
|      | +hv       | (0.42)    | <240           | Photolysis products |
|      | +hv       | (0.42)    | <240           | Photolysis products |
|      | DTT       | 0.42      | 255            | Photolysis products |
|      | 3'-Nucleotidase | NC | NC | Reduced product |
| 3    | None      | 0.60      | 278            | ND |
|      | +hv       | (0.55)    | <240           | Photolysis products |
|      | +hv       | (0.55)    | <240           | Photolysis products |
|      | +hv       | (0.55)    | <240           | Photolysis products |
|      | DTT       | (0.50)    | 255            | Photolysis products |
|      | 3'-Nucleotidase | NC | NC | Reduced product |
| 4    | None      | 0.24      | 288-274        | Breakdown products |
|      | +hv       | NC        | NC             | Breakdown products |
|      | +hv       | NC        | NC             | Breakdown products |
|      | +hv       | NC        | NC             | Breakdown products |
|      | DTT       | NC        | NC             | Breakdown products |
|      | 3'-Nucleotidase | NC | NC | Breakdown products |
| 5    | None      | 0.20      | 259            | [γ-32P]ATP |
|      | +hv       | NC        | NC             | [γ-32P]ATP |
|      | +hv       | NC        | NC             | [γ-32P]ATP |
|      | +hv       | NC        | NC             | [γ-32P]ATP |
|      | DTT       | NC        | NC             | Breakdown products |
|      | 3'-Nucleotidase | NC | NC | Breakdown products |
| 6    | None      | 0.37      | 278            | [5',32P]p8N3G3'p |
|      | +hv       | (0.24; 0.72) | >240 | Photolysis products |
|      | +hv       | (0.24; 0.72) | >240 | Photolysis products |
|      | +hv       | (0.24; 0.72) | >240 | Photolysis products |
|      | DTT       | 0.31      | 278            | Breakdown products |
|      | 3'-Nucleotidase | 0.54 | 278 | [5'-32P]p8N3G3'p |
|      | DTT + 3'-nucleotidase | 0.42 | 255 | [5'-32P]p8N3G3'p |

* Treatments were as follows: +hv, photolysis for 5 min as described in the legend to Fig. 1; DTT, reduction with DTT, pH 7.5, as shown in Fig. 3; 3'-nucleotidase, 2-min incubation with 0.1 unit of enzyme in 50 μl of 10 mM Tris, pH 7.0, and 0.4 M Zn(OAc)2 solution.

\( R_F \) values were obtained using PEI-cellulose TLC plates developed in 0.38 M KH2PO4, pH 3.4, solvent. Values in parentheses indicate smeared spots.

Products were identified by analysis of their UV spectra, photoactivity, reduction with DTT, enzyme interactions, and \( R_F \) values as compared to known standards.

G-6-P, glucose-6-P; NC, no change in treatment; ND, not determined.

Blue-stained bands, while p65 is a relatively minor component.

In an attempt to obtain an apparent \( K_d \) for [5'-32P]p8N3Gp binding, levels of photoincorporation at the various concentrations were determined by cutting out the band from the gel and measuring the amount of radioactivity by scintillation counting. As can be seen from Fig. 6, p65 shows saturation, with an apparent \( K_d \) of 10 μM. The other protein, p86, does not show saturation, indicating a \( K_d \) in excess of 10 μM. Although p86 has a lower affinity for the analog than does p65, the specificity of the interaction is indicated by two observations. First, p86 is photolabeled at very low concentrations of analog (5 μM); and, second, p86 is one of only two major bands even at 30 μM [5'-32P]p8N3Gp. There was no detectable radioactivity incorporated by a sample incubated with the [5'-32P]p8N3Gp but not photolyzed or incubated with prephotolyzed analog. This indicates that the radioactive labeling is photodependent.

The autoradiograph of Fig. 7 shows the results of initial competition experiments. In this type of experiment, various nucleotides in addition to the photoactive nucleotide are added to the sample. If the nucleotide binds to the same site as the analog, the photoincorporation resulting from photolysis will be decreased. The greatest decrease in radioactivity will occur when the tightest-binding ligand is present. Thus, if the [5'-32P]p8N3Gp binds to a GTP site, one would expect...
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The use of 8-azidopurine photoprobes of various 5'-phosphorylated adenosine- and guanosine-containing nucleotides has proven to be quite successful. These probes have been used to identify specific nucleotide-binding proteins of cAMP, cGMP, ATP, GTP, and S-adenosylmethionine in biological systems of varying complexity. They have also been used to resolve certain mechanistic aspects of nucleotide-regulated phenomena (see Refs. 15, 19, and 20 and references therein).

One of the main advantages of this type of photoprobe is that they may be used to detect and study specific nucleotide-protein interactions in complex systems. They may also be used as molecular markers to aid in the purification as well as identification of the biochemical properties of specific proteins. In the same way, photoprobes of the (p)ppGpp homologous series may be used to localize specific 5'p,Gpn binding proteins in subcellular fractions (membrane, cytosol, or DNA-binding proteins); to examine the modulation of enzyme activity by alterations in 5'p,Gpn-protein interactions caused by other nucleotides or environmental factors such as pH, ionic strength, and ionic composition; and to monitor purification of these proteins.

The synthesis of $[5'-32P]p8N_3Gp$ presented two serious problems, one involving the behavior of the azido group, and the other the chemistry of the 3'-phosphate bond (see Fig. 3).

It is difficult to prevent destruction of the azido moiety of the $[5'-32P]p8N_3Gp$. This difficulty stems from many sources. 1) The azido group is thermally labile—prolonged exposure to elevated temperatures converts $[5'-32P]p8N_3Gp$ to nonphotoactive compounds (one of which is p8NH2Gp). Therefore,
concentration of azido nucleotide solutions, by rotary evaporation for example, must be done at low temperatures (18 °C or lower). 2) The analog is readily reduced to p8N3Gp by the presence of even very low concentrations of certain reducing reagents, especially DTT. The 8-azidoguanosine analogs also appear to be reduced slowly by cysteine and mercaptoethanol under the conditions used for the polynucleotide kinase reaction. Unfortunately, this enzyme requires a reducing agent and comes in a buffered solution containing mercaptoethanol (cautionary note; some companies use DTT instead). 3) Extremes of pH are deleterious to the stability of the azido and exacerbate the problems posed by temperature and reducing agents. The enzymatic synthesis described in this report involves exposure to certain levels of these undesirable conditions: the reaction temperature is 37 °C, mercaptoethanol is present, and the pH for optimum polynucleotide kinase activity is 8.9. The temperature and/or the pH may be decreased, but the more amenable conditions decrease enzyme activity; therefore, increased reaction time is required with consequent increased exposure to the reducing agent. The problem with the 3'-phosphate arises because extremes of pH catalyze the exchange of the phosphate between the 2' and 3'-hydroxyls. Gp and BrGp are exposed to pH 3.4 during the bromination procedure and some BrG2'p may be formed, but this presents no problem since the final product, 8N3Gp, is not a substrate for the polynucleotide kinase. The pH 8.9 environment during the enzymatic synthesis of [5'-32P]p8N3Gp probably causes the observed transesterification. As mentioned above, the pH can be decreased, but then the increased reaction time may result in increased reduction of the azido group. Despite these problems, the [5'-32P]p8N3Gp may be obtained in reasonably pure form and seems to be an effective photoaffinity analog of Gp. As such, it interacts with proteins that also bind ppGpp and ppGp, which are proposed to be regulatory nucleotides.

During normal growth, E. coli maintains a basal level of ppGpp in the low micromolar range; during nutritional stress, the stringent response occurs and ppGpp concentrations may rise to millimolar levels (21). It has also been suggested that basal concentrations of this analog may have a regulatory role (23). Another magic-spot compound, ppGp, is produced under certain circumstances and it, too, may have a role in regulation of biochemical processes such as RNA transcription (9–11).

The ppGpp that is synthesized generally seems to exert its action through a number of regulatory proteins, which either bind ppGpp and/or ppGp, and possibly by ppGp. The analog used in this research, [5'-32P]p8N3Gp, fulfills this criterion with respect to p8N3Gp, as shown in Fig. 7. The photolabeling of p86 with [5'-32P]p8N3Gp is about equally inhibited by GTP, ppGpp, and ppGp, but pGpp has no apparent effect. GTP and magic-spot nucleotides apparently compete for the same site, but the nucleotide ppGpp, which is not believed to be a biological effector, has no effect on photoincorporation of the analog. Although no Kd could be determined from the photolabeling data (since concentrations of analog higher than 30 μM would lead to too much nonspecific labeling), the fact that this protein was labeled at such low analog concentrations indicates that the Kd would probably be under 50 μM.

Photoincorporation into p65 is best reduced by ATP and GMP; GTP, ppGpp, and ppGp are less effective. Since ATP is the most effective competitor, it is possible that the analog is labeling an ATP site. However, one might expect the high affinity binding of the analog to correlate with a corresponding specificity. It is probable that ATP allosterically modulates [5'-32P]p8N3Gp binding or that ATP phosphorylation causes the decreased photolabeling of p65 and the increased labeling of p86. Our recent data indicate that [γ-32P]ATP (and GTP) phosphorylates both of these proteins and that ATP phosphorylation may be the cause of the variations in [5'-32P]p8N3Gp photoincorporation. Why GMP should decrease photoincorporation into p65 is at present unclear. However, the data are not inconsistent with both GMP and ppGp binding to this protein in vivo, in which case, the observed GMP competition at 500 μM against 5 μM analog would not be surprising.

In summary, under the conditions employed in this study, the analog [5'-32P]p8N3Gp photolabels primarily two proteins in E. coli sonicates. Differential protection against photoincorporation was observed with various nucleotides that one would expect to interact with p8N3Gp-binding sites. Although the identity of neither of these bands is known, the use of the photoaffinity probe [5'-32P]p8N3Gp (and also [γ-32P]p8N3Gp) can be used to characterize these proteins. The analog can also be used to study purified proteins, e.g., enzymes known to interact with ppGpp. One such enzyme, RNA polymerase, is inhibited both reversibly and irreversibly (on photolysis) by micromolar levels of ppGpp. In addition, [5'-32P]p8N3Gp (or the "cold" nucleotide) may also be used to prepare [32P]ppN3Gp and [32P]ppGp by the chemical addition of phosphate (e.g., see Ref. 24). These probes would provide other valuable information concerning the regulatory effects of these nucleotides.

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