COMMUNICATIONS

Kinetics of Coupling Reactions That Generate Monothiophosphate Disulfides: Implications for Modification of RNAs

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The inclusion of guanosine-5′-monothiophosphate (GMPS) in an in vitro transcription reaction facilitates enzymatic synthesis of an RNA transcript with a monothiophosphate group at the 5′ end. A kinetic study of the modification reactions that generate monothiophosphate disulfide linkages with either 5′-GMPS alone or 5′-GMPS-primed RNA as the substrate revealed that the second-order rate constants increased as the pH was decreased. For example, when the reaction pH was lowered from 8 to 4, the k_2 value for the coupling reaction between N-(6-[biotinamido]hexyl)-3′-(2′-pyridyldithio)-propionamide (biotin-HPDP) and GMPS increased 67-fold from 1.84 to 123 M^{-1} s^{-1}. In addition to discussing a possible mechanism for coupling reactions that involve GMPS and disulfides, we also indicate conditions that are likely to be optimal for modification of the nucleophilic sulfur in 5′-GMPS-primed RNAs.

INTRODUCTION

Impetus for the design of simple strategies for site-specific incorporation of biochemical and biophysical probes in RNAs is fueled by the increasing realization that the role of RNA and RNA−protein interactions is pervasive in various cellular and developmental processes. Advances in solid-phase oligonucleotide synthesis of small RNAs have facilitated the site-specific introduction of functional groups that lend themselves to subsequent chemical modification. For instance, a 2′ amino group in an RNA oligonucleotide can be converted to a thiol (by treatment with an activated ester and a reducing agent), which in turn can be modified to incorporate a probe of choice (*4*). However, for large RNAs that cannot be synthesized chemically, a commonly employed approach has been to include guanosine-5′-monothiophosphate (GMPS) in an in vitro transcription reaction to enable enzymatic synthesis of an RNA transcript whose 5′ end contains the monothiophosphate group (*10*). The nucleophilic sulfur at the 5′ end can be subsequently modified to introduce a photoaffinity cross-linking agent or a spin label or an affinity tag (*3, 7, 9*). Despite recent reports of successful modifications of 5′-GMPS-primed RNAs, the kinetics and mechanism of the coupling reactions remain uncharacterized. In this report, we have examined the kinetics and pH-dependence of the modification reactions that generate monothiophosphate disulfide linkages using either 5′-GMPS or 5′-GMPS-primed RNA as the substrate.

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RESULTS AND DISCUSSION

Spectrophotometric measurement of the kinetic rates for the coupling reactions with GMPS was made possible by choosing 2-thiopyridyl derivatives (such as 2-PDS and biotin-HPDP, Figure 1) as modifiers. The modification reactions were carried out at 24 (±2) °C under pseudo-first-order conditions with the pyridine disulfide in at least 30-fold excess. Plots of log[GMPS] versus time were linear over two half-lives and the calculated second-order rate constants (k_2) did not exhibit any significant variation when the concentrations were varied over a 3-fold range. When k_2 values were examined as a function of pH, an increase in the rate was observed as the pH of the reaction was decreased (Figure 2). For instance, the second-order rate constant for the reaction between GMPS and 2-PDS in 20% DMF (v/v) showed an 18-fold increase as the pH was decreased from 8 to 5.8 (Figure 2). Similarly, the coupling reaction between biotin−HPDP and GMPS was accelerated 67-fold (from 1.84 to 123 M^{-1} s^{-1}) as the reaction pH was lowered from 8 to 4 (Figure 2).

These findings with GMPS as a substrate prompted us to pursue the biotin−HPDP modification reaction with a 5′-GMPS-primed precursor tRNA (ptRNA). The rate of biotinylation of 5′-GMPS-primed ptRNA was enhanced 2 orders of magnitude upon lowering the pH from 8 to 4 (Figure 2). However, since this conclusion is based on 2-thiopyridone release and not based on direct quantitation of biotin content in the RNA, we decided to employ an independent measure of biotinylation. The modification reaction was performed at different pH values, and the putative biotinylated ptRNAs were transferred to a nylon membrane wherein they were probed with a streptavidin−horseradish

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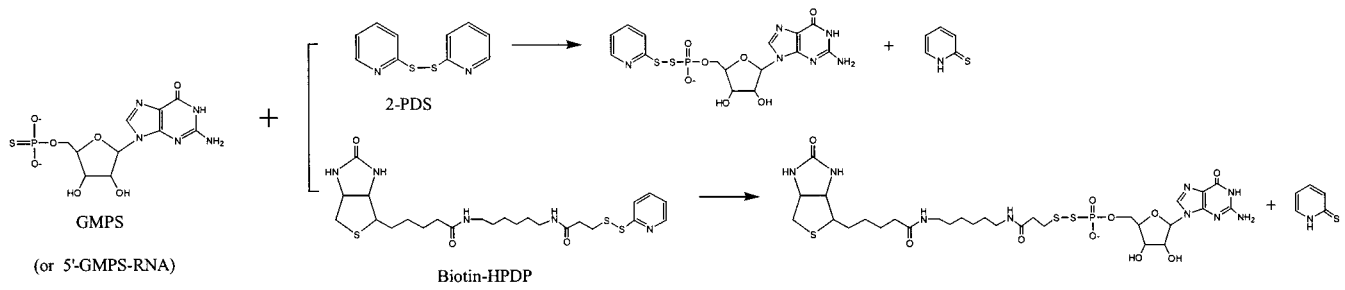

Figure 1. Generation of disulfide linkages using GMPS as the substrate. The coupling reactions between GMPS (synthesized as described elsewhere (*1*)) and either 2,2′-pyridine disulfide (2-PDS, Sigma) or N-(6-[biotinamido]hexyl)-3′-(2′-pyridyldithio)-propionamide (biotin−HPDP, Pierce) are illustrated. A variation on the latter reaction involves the modification of a 5′-GMPS-primed RNA with biotin−HPDP. The ptRNA^{Gln} substrate was synthesized in vitro by incubating 38 μg/mL BstN I-linearized pUC-pGln (*8*) with 4.8 mM GMPS, 1.2 mM GTP, 6 mM ATP, 6 mM CTP, 6 mM UTP, and 400 U T7 RNA polymerase (Ambion) for 5 h at 37 °C in 200 μL of 200 mM HEPES, 40 mM DTT, 2 mM spermidine, and 28 mM $MgCl_2$. After DNase I treatment and phenol/chloroform extractions, the RNA in the aqueous phase was washed over a Microcon YM-10 membrane to remove unutilized rNTPs and GMPS.

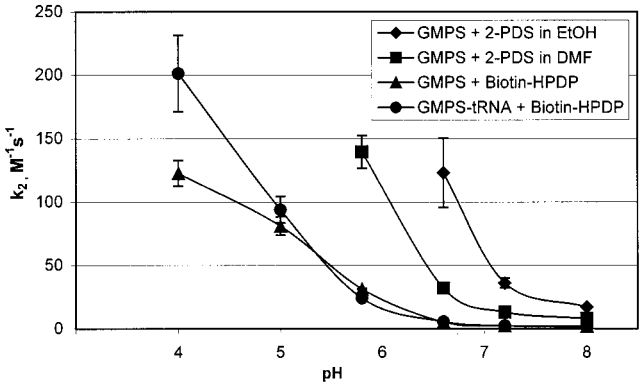

Figure 2. The pH dependence of second-order rate constants for the various GMPS-coupling reactions (see text for details). Inset provides a key to the symbols used in the figure. The averages were calculated using second-order rate constants obtained from at least two independent experiments. 2-PDS and biotin−HPDP were dissolved in either 50% (v/v) ethanol or DMF and diluted appropriately for the modification reactions. The reactions of GMPS with either 2-PDS or biotin−HPDP were performed in 0.1 M phosphate or citrate or acetate buffer. Reactions were initiated by rapid mixing (Sigma cuvette mixer) of stock GMPS. Kinetics of the coupling reactions were measured using a BioSpec-1601 spectrophotometer (Shimadzu) by following the appearance of 2-thiopyridone at 343 nm (using $\epsilon = 7060$ M^{-1} cm^{-1}) for reactions in either 20% (v/v) ethanol or 20% (v/v) DMF.

peroxidase (HRP) conjugate. Increased biotinylation of ptRNA with decreasing pH would result in greater binding of the HRP-linked streptavidin. In the presence of a chemiluminescent substrate for HRP, the signal produced would be proportional to the amount of RNA that was biotinylated. The chemiluminescence signal is at least 5-fold more intense when the GMPS-primed ptRNA was modified with biotin−HPDP at pH 4 than at pH 8 (Figure 3). Since a 70-min incubation period was used for these ptRNA modification reactions, the 100-fold difference in second-order rate constants between pH 4 and 8 manifests itself as a much smaller (5-fold) apparent difference between the two pH values (Figures 2 and 3). Although this explanation could be validated by performing the tRNA modification reaction for a much shorter time (e.g., only 5 min), this was not technically feasible due to the inability to terminate the reaction prior to loading the products on the polyacrylamide gel.

The pH-rate profiles for the reactions that we have studied provide some clues with regard to the mechanism of the GMPS-modification reactions. The reactions are first-order in hydrogen ion concentration over the pH range that we studied. The first pK_a of GMPS is not known with accuracy, but it is probably around zero; the second pK_a is about 5.2 (*6*). The first pK_a for 2-PDS is 0.35 and the second pK_a is 2.37 (*5*). Since the rates of the coupling reactions are very slow at pH 7 and above (Figure 2), a significant reaction between the dianionic form of GMPS and the neutral form of 2-PDS (or similar 2-thiopyridyl derivatives) is excluded. The rates do increase remarkably with a decrease in pH and suggest that the most significant contribution to the reaction rate is probably due to the GMPS monoanion attacking either (or both) the monocation or the dication of 2-PDS. Moreover, the increased rates at acidic pH values could be attributed to the fact that protonation of both the ring nitrogens in 2-PDS not only enhances the electrophilic nature of both the sulfurs but creates a better leaving group in the protonated thiopyridone (*2*, *5*).

It is instructive to compare the predominant pathways for the reaction of 2-PDS with either a thiol (e.g., 2-mercaptoethanol) or GMPS. The former reaction involves the nucleophilic attack of the thiolate anion on the unprotonated disulfide at pH values near and above neutrality. As expected, the pH−rate profile reveals that when the pH is decreased from pH 10 to pH 5, the rate decreases sharply probably due to a reduction in the thiolate anion concentration (*2*, *5*). However, between pH 5 and 1, a plateau is observed in the pH−rate profile. In this pH range, protonation of the pyridine rings results in enhanced reactivity of 2-PDS and offsets the drop in rate due to decreased thiolate concentration. Although the increased electrophilicity of the protonated 2-PDS prevents the rate from falling further, it is unable to cause an overall increase in the rate; hence, the plateau. In contrast, we find that the reaction of 2-PDS with GMPS displays an increase as the pH is decreased from 8 to 4 (Figure 2). This is due principally to the increased electrophilicity of 2-PDS at acidic pH values.

A practical outcome of this study is the optimization of reaction conditions for modifications involving 5′-GMPS-primed RNAs and 2-thiopyridyl derivatives coupled to different probes. While others have performed GMPS-coupling reactions at near-neutral pH values and succeeded in ∼70% modification (*9*), we believe that by

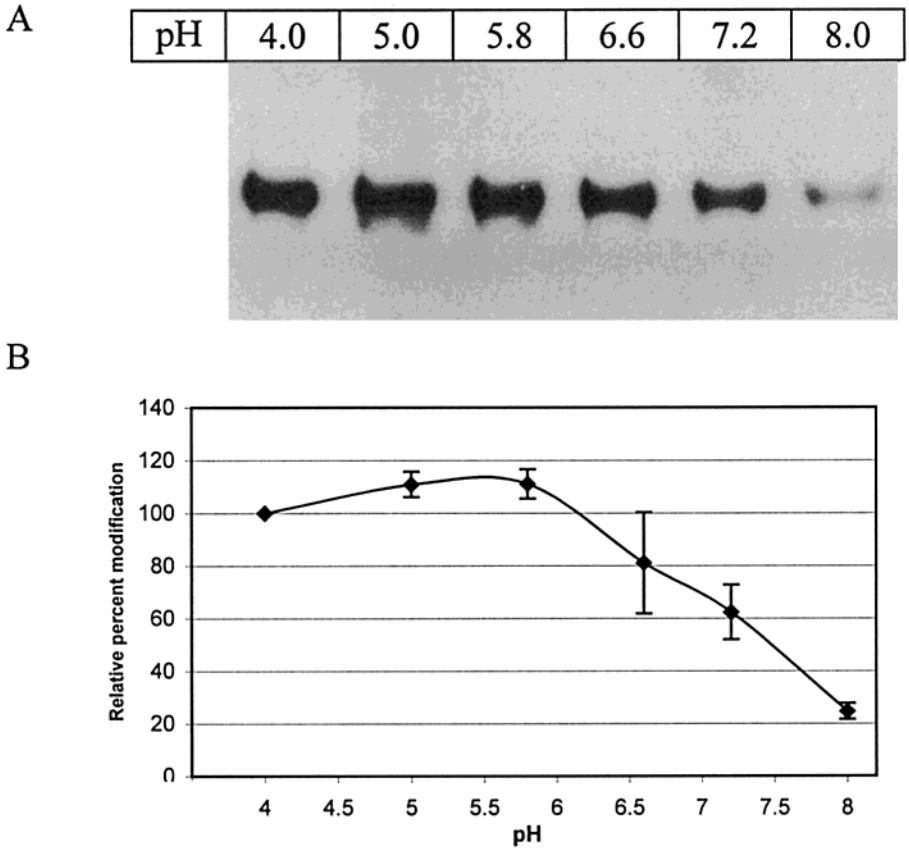

Figure 3. RNA modification and Northern blot analysis. Panel A, the pH dependence of biotinylation of GMPS-primed tRNA as detected by chemiluminescence (see below). Panel B, quantitation of the data shown in panel A. Multiple reactions (20 μL each) were set up with 10 μM GMPS-primed tRNA and 300 μM biotin−HPDP in 0.1 M buffer of varying pH in the range 4 to 8. The RNA samples were then electrophoresed in a 7 M urea/ 10% polyacrylamide gel and electroblotted to a Zeta-probe membrane (Bio-Rad). After blocking the membrane for 1 h in 50 mL of nonfat dry milk [5% (w/v)]-supplemented TBST buffer (10 mM Tris, 150 mM NaCl, 0.1% Tween 20), the membrane was washed thrice with TBST and incubated for 1 h in 30 mL of TBST containing 30 μL of 0.1 mg/mL peroxidase-conjugated streptavidin. After washing the membrane thrice in 50 mL of TBST, 3 mL of luminol/enhancer solution (SuperSignal West Dura Extended Duration Substrate) was added directly to the membrane for 1 min. The membrane was exposed to an X-ray film to obtain the photographic record displayed in Panel A. The chemiluminescence generated by the peroxidase was quantitated by using a FluorChem 8000 (Alpha Innotech) to obtain the data shown in Panel B.

lowering the pH of the reaction, the efficiency can be easily made to approach 100% and the reaction time can be shortened considerably (e.g., 5 min at pH 5 instead of 70 min at pH 8). Moreover, longer incubations at high pH are undesirable since both the 2-thiopyridyl substrates and the monothiophosphate disulfide product are unstable at alkaline pH values (*9*).

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LITERATURE CITED

(1) Behrman, E. J. (2000) An improved synthesis of guanosine 5′-monothiophosphate. *J. Chem. Res. S* 446−447.

(2) Brocklehurst, K., Stuchbury, T., and Malthouse, J. P. (1979) Reactivities of neutral and cationic forms of 2,2′-dipyridyl disulphide towards thiolate anions. *Biochem. J. 183*, 233−238.

(3) Burgin, A. B., and Pace, N. R. (1990) Mapping the active site of ribonuclease P using a substrate containing a photoaffinity reagent. *EMBO J. 9*, 4111−4120.

(4) Cohen, S. B., and Cech, T. R. (1997) Dynamics of thermal motions within a large catalytic RNA investigated by cross-linking with thiol-disulfide exchange. *J. Am. Chem. Soc. 119*, 6259−6268.

(5) Grimshaw, C. E., Whistler, R. L., and Cleland, W. W. (1979) Ring opening and closing rates for thiosugars. *J. Am. Chem. Soc. 101*, 1521−1532.

(6) Jaffe, E. K., and Cohn, M. (1978) ^{31}P nuclear magnetic resonance spectra of the thiophosphate analogues of adenine nucleotides; effects of pH and Mg^{2+} binding. *Biochemistry 17*, 652−657.

(7) Mackosko, J. C., Pio, M. S., Tinoco, I., Jr., and Shin, Y.-K. (1999) A novel 5′-displacement spin-labeling technique for electron paramagnetic resonance spectroscopy of RNA. *RNA 5*, 1158−1166.

(8) Pascual, A., and Vioque, A. (1999) Substrate binding and catalysis by ribonuclease P from cyanobacteria and *Escherichia coli* are affected differently by 3′ terminal CCA in tRNA precursors. *Proc. Natl. Acad. Sci. (U.S.A.) 96*, 6672−6677.

(9) Sengle, G., Jenne, A., Arora, P., Seelig, B., Nowick, J. S., Jaschke, A., and Famulok, M. (2000) Synthesis, incorporation efficiency, and stability of disulfide bridged functional groups at RNA 5′-ends. *Bioorg. Med. Chem. 8*, 1317−1329.

(10) Wecker, M., and Smith, D. (2000) Selection for RNA: peptide recognition through sulfur alkylation chemistry. *Methods Enzymol. 318*, 229−237.

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