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Cytosine nucleoside inhibition of the ATPase of *Escherichia coli* termination factor rho: evidence for a base specific interaction between rho and RNA

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ABSTRACT

The function of rho factor in transcription termination depends on interactions with nascent RNA molecules that contain unpaired cytidylate residues. We show that cytidine, as a free nucleoside, inhibits the binding of rho to λ *cro* mRNA and is a competitive inhibitor of rho-ATPase activity with λ *cro* mRNA as cofactor. The relative ability of various cytidine analogs and other nucleosides to inhibit the rho-RNA interaction was used to probe features responsible for the base specificity of rho action. The results suggest that rho has a specificity pocket in its polynucleotide-binding site that apparently can make H-bond interactions with the side of the cytosine ring that normally faces away from the sugar ring and that may involve a relatively close fit along the edge of the ribose ring at the C_2' carbon. The nature of the complex of rho with cytidine nucleotides was analyzed further by determining whether incubation with BrCMP caused inactivation of rho ATPase. Although BrCMP could form Michaelis inhibition complexes, it did not activate rho. Rho thus lacks a diagnostic property of enzymes that make specific covalent addition complexes with pyrimidines.

INTRODUCTION

Transcription termination factor rho from *Escherichia coli* mediates release of RNA from transcription complexes (1) in a reaction process that is initiated by the binding of rho to the nascent RNA and is driven by the hydrolysis of ATP (2–6). Rho protein also catalyzes hydrolysis of ATP with free, isolated RNA transcripts as cofactors (7,8). Since the termination and rho-ATPase activities have the same dependence on RNA sequence and structure and the same sensitivities to reaction conditions, the ATP hydrolysis reaction with free RNA serves as a convenient monitor for mechanistic studies of rho action on nascent transcripts.

One of the requirements for activation of rho ATPase is the presence of unpaired cytidylate (C) residues in the RNA cofactor (9). RNA molecules lacking C residues have virtually no cofactor activity, while poly(C) and synthetic copolymers of U and C containing as few as one C residue out of 20 are very potent activators. These requirements suggest that rho protein may have a site or set of sites that can interact directly with C residues. Since rho binds to poly(C) with high affinity even in the presence of high concentrations of monovalent counterions (*i.e.*, 2M KCl), the interactions in a putative C-specific site are likely to involve non-ionic bonds with the base and sugar components of a C residue (10). Hence, such a site could bind cytidine directly as a free nucleoside.

We report here evidence for the interaction between rho and cytidine and characterize some of the features responsible for the specificity of its binding site from the ability of various cytidine analogs to reproduce the action of cytidine. The results indicate that determinants on the C_2-N_4-C_4 side of the cytosine base and on the C_2' of the ribose group are important in the binding of RNA to rho.

MATERIALS AND METHODS

Biochemicals and enzymes

Nucleoside triphosphates were purchased from Boehringer Mannheim Corp. [α-^{32}P]UTP (3200 Ci/nmol) and [α-^{32}P]ATP (650 Ci/nmol) were obtained from ICN Chemical and Radioisotope Division. All other nucleosides and nucleotides were purchased from Sigma Chemical Co. Rho protein was isolated from AR120(p39-AS) as described by Mott *et al* (11). T7 RNA polymerase was isolated from BL21(pAR1219) (12) by the procedure of Tabor and Richardson (13).

RNAs

The methods for synthesis and purification of λ *cro* RNA are described in Faus and Richardson (8). The RNA in all cases was transcribed from pIF2 DNA that had been digested with *Taq*I; it thus consists of a transcript of the *cro* gene to nucleotide 378.

ATPase assays

ATP hydrolysis was measured by the release of labeled ADP from [α-^{32}P]ATP as follows: The standard reaction mixture with *cro* RNA as cofactor contained 0.34 pmol (95 ng) of rho and 0.54 pmol (68 ng) of *cro* RNA in 20 μl of a solution

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consisting of 40 mM Tris-HCl (pH 7.9), 25 mM KCl, 1 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.1 mM EDTA and 1mM [α-^{32}P]ATP (800 cpm/nmol); it was incubated for 45 min at 37°C. The hydrolysis of ATP by rho-RNA complexes was strictly linear for at least 60 min, as long as less than 80% of the initial amount of ATP was hydrolyzed. The 45 min time point was chosen to measure the rates, as it gives an adequate level of ATP hydrolyzed with the low level of rho used. The standard reaction mixture with poly(C) as cofactor contained 0.22 pmol (60 ng) of rho and 50 ng of poly(C) (150 pmol of CMP residues) in TKM buffer [50 mM tris-HCl (pH 8.0), 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA] with 1 mM [α-^{32}P] ATP (~ 1,000 cpm/nmol); it was incubated for 10 min at 37°C. For both, the reaction was quenched by addition of EDTA to 50 mM and ADP to 80 mM and a 4 μl sample was applied to a poly(ethylenimine)-cellulose strip (Brinkmann), which was then developed with 1 M formic acid-0.5 M LiCl to separate ADP from ATP. The ADP and ATP spots, located with an ultraviolet light, were cut from the strip, and the strip segments were placed in separate vials with 2 ml of a toluene-based scintillation mixture and assayed for radioactivity.

Rho-RNA binding assays

Binding reaction mixtures contained 10.7 fmol of [^{32}P] *cro* RNA and 17.5 fmol of rho in 50 μl of binding buffer [40 mM Tris-HCl (pH 8.0) 25 mM KCl, 1mM $MgCl_2$, 0.1 M mM dithiothreitol, 0.1 mM EDTA] containing 0.25 mg of acetylated bovine serum albumin/ml and nucleosides as indicated. After incubation for 2 min. at 37°C, the solution was filtered through a 25 mm Schleicher & Schuell BA85 nitrocellulose filter. Each filter was washed twice with 0.25 ml portions of binding buffer, dried, and assayed for radioactivity as described for the ATPase assays. Further details of the procedure along with controls that indicate its validity are presented in Ceruzzi *et al.* (14) and in Faus and Richardson (8).

RESULTS AND DISCUSSION

Cytidine competes with an mRNA for binding to rho

Several properties of the interaction between rho and an RNA transcript have been established for λ *cro* RNA (8,14). One property that correlates well with the function of rho factor in transcription termination at λtR_1 is the activation of rho-ATPase with isolated λ *cro* RNA as a cofactor. Perturbation of that interaction can be analyzed readily by standard, steady-state enzyme kinetic methods. Thus, if cytidine binds to the C-specific domain of the RNA binding site, it should act as a competitive inhibitor of RNA cofactor function in activation of ATP hydrolysis. Figure 1 shows the effects of cytidine and of uridine on rho-ATPase with λ *cro* RNA as a cofactor in slight stoichiometric excess over rho hexamer. In the absence of cytidine, rho catalyzed the hydrolysis of ATP at a rate of 650 molecules-min^{-1} per rho hexamer (2.3 nmol-min^{-1}-μg^{-1}). That rate was reduced by 50% and 80% with 20 mM and 100 mM cytidine, respectively, but was reduced by less than 5% with 100 mM uridine. Thus between these two pyrimidine nucleosides, inhibition was selective for cytidine. To test whether inhibition is competitive with λ *cro* RNA, rates of ATP hydrolysis without or with 30 mM cytidine present were measured at various concentrations of λ *cro* RNA and the data analyzed on a double reciprocal plot. The results (Figure 2) show that the rates of hydrolysis, as indicated by the values of 1/v, were the same in

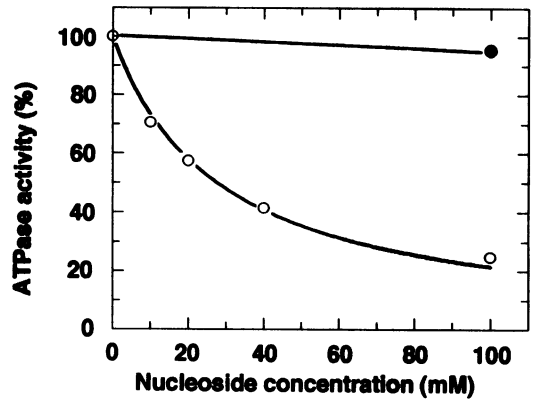

Figure 1. Inhibition of rho-λ cro mRNA ATPase activity by cytidine. ATP hydrolysis was measured as described in Materials and Methods with reaction mixtures containing λ cro mRNA and the indicated concentrations of cytidine (○) or uridine (●). The amount of ATP hydrolyzed in the reaction mixtures with no nucleoside, the 100% activity value, was 9.8 nmoles. The curve that connects the points with cytidine is the best fit to the equation:

$$\frac{V_{max} \times [S] \times 100}{V \times (K_m(1 + \frac{[i]}{K_i}) + [S])}$$

using the values of V_{max} and Km determined in Figure 2. The K_i determined from this best fit was 11.1 ± 0.6 mM, which is in good agreement with the K_i determined independently in Figure 2.

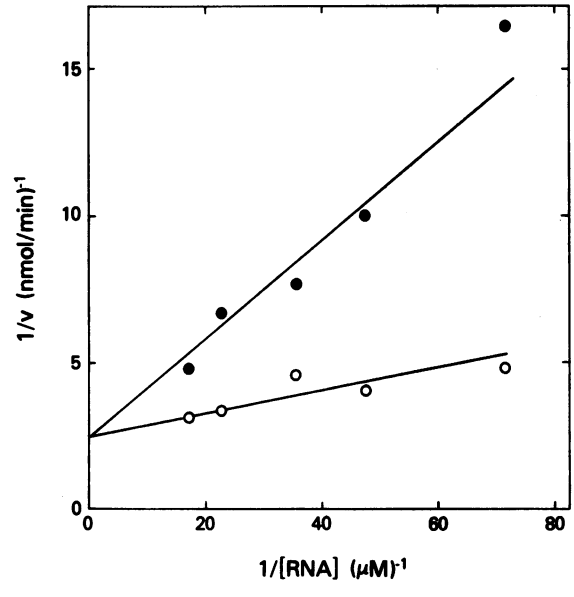

Figure 2. Cytidine competes with RNA for inhibition of rho-ATPase. Rates of ATP hydrolysis (v) were determined with standard reaction mixtures containing the indicated concentrations of λ cro mRNA with (○) or without (●) 30 mM cytidine. The rate values are for the 95 ng of rho in the reaction mixture.

the presence or absence of inhibitor upon extrapolation to infinite cofactor concentration (1/[RNA] = 0). Thus, cytidine competes with *cro* RNA to inhibit rho-ATPase with K_i = 10 mM.

The inhibition of the interaction between rho and λ *cro* RNA can also be detected with direct binding assays. The assays we used measure the retention of labeled λ *cro* RNA with rho protein

Table 1. Inhibition of rho binding by nucleosides.

Compound	Concentration for 50% inhibition[a] (mM)	Compound	Concentration for 50% inhibition[a] (mM)
cytidine	20	5-bromocytidine	40
2'-deoxycytidine	8	azacytidine	10
arabinosylcytosine	46	2-thiocytidine	>>40[b]
5'-CMP	20	N,N-dimethyl -2'-deoxycytidine	>>50[b]
3'-CMP	10	α-cytidine	>>100
2'-CMP	35	uridine	>>100
3'-O-methylcytidine	32	adenosine	>>100
2'-O-methylcytidine	>>100		

[a]The values were all determined from binding inhibition curves and have an error of ± 10%. For each compound that did not inhibit binding significantly (less than 5% at the highest concentration tested), the value is given as much greater than (>>) twice the highest concentration tested. [b]The highest concentration tested was limited for these by their low solubility.

on membrane filters (8,14). With a limiting amount of RNA (0.21 nM), the presence of 0.35 nM rho caused 63% of the labeled λ *cro* RNA to be retained on the filter with our standard conditions in the absence of cytidine. However, in the presence of 20 mM cytidine, the amount of RNA retained was half that value (Table 1). Thus, the results of the direct RNA binding assays confirm those of the ATPase inhibition studies.

Although the inhibition with cytidine is specific and competitive with RNA, it is relatively weak; the K_i for cytidine is about 10^6 fold higher than the K_d for the rho-*cro* RNA interactions. However, this difference is reasonable considering that the RNA site within a single subunit of rho likely consists of a relatively large surface that can make multiple contacts with several residues on a single RNA molecule with the cytidine specificity pocket being only part of that surface. Thus, the RNA molecule presumably forms a large number of weak bonds over several subunits in the hexameric rho protein, whereas a single cytidine molecule binds only to a part of the binding surface on a single subunit.

Since the action of rho in termination is dependent upon its binding to sites on the nascent transcript, we tested whether cytidine could inhibit rho action at tR_1, the terminator for λ *cro* RNA. However, the addition of 20 mM cytidine had no effect on normal rho action during transcription *in vitro* of a *Hinf* I-λ DNA fragment containing the sequences of P_R and tR_1 (data not shown). One possible explanation for this negative result is that rho may be interacting with the RNA as part of a preformed complex with RNA polymerase in the transcription complex. In this case, the binding of the nascent RNA will be quasi-unimolecular, and the local concentration of the nascent RNA in the domain of rho factor bound to RNA polymerase would be effectively much higher than that of free RNA at the same overall concentration. Since the binding of cytidine is competitive with RNA, a higher effective concentration would allow the RNA to compete better. The limitation on the solubility of cytidine precluded testing a much higher concentration.

Inhibition with cytidine analogs

Because of its simplicity, the direct binding assay can be used to screen a large number of cytidine analogs and other nucleosides for their effectiveness in inhibiting the binding of rho to λ *cro* RNA. The differences in effectiveness can then be correlated with the structural change in the nucleoside to delineate the features

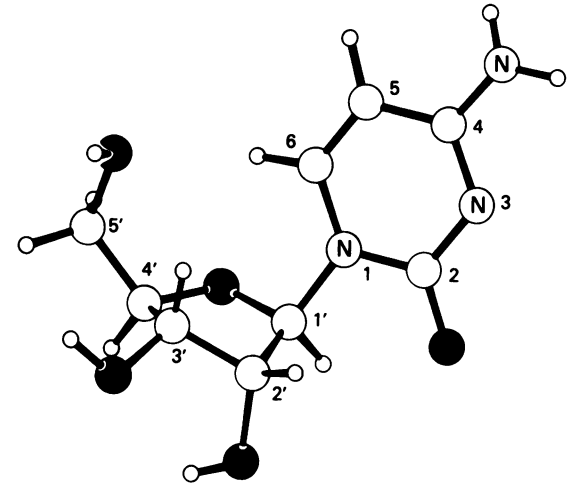

Figure 3. A perspective drawing of cytidine. (○) H atoms, (○) C atoms, N atoms, (●) O atoms.

of the cytidine residues that are responsible for the binding interaction. The results (Table 1) indicate that functional groups on both the pyrimidine ring and the sugar ring contribute to the effectiveness of cytidine as an inhibitor. Figure 3 shows a perspective drawing of cytidine that is based on its crystal structure (15). We use this structure to formulate a working model of the possible disposition of functional groups that are responsible for the binding of cytidine residues in rho's RNA binding site. In agreement with the ATPase assay results, uridine did not inhibit binding of rho to *cro* RNA (Table 1). This suggests that binding specificity is determined by having a good H-bond donor group on position 4 or an H-bond acceptor at position 3 (the imino N) or both. The finding that N,N-dimethyl-2'-deoxycytidine failed to give significant inhibition (the 2' hydroxyl group is not a specificity determinant, as noted below) indicates that an H-bond donor group on position 4 is a critical element.

Another change on the pyrimidine ring that greatly affected competition was replacement of the oxygen at C_2 with S; 2-thiocytidine had negligible inhibition when tested at the limit of its solubility (Table 1). This chemical change has several consequences (16): the van der Waals radius is somewhat larger

for S than for O (1.85 Å vs. 1.4 Å); S is a poorer H-bond acceptor than is O, and the C=S double bond is about 0.4Å longer than is the C=O double bond. Any or all of these could contribute to a poorer fit and/or weaker bonding interactions. In a crystal, the pyrimidine base in cytidine has the *anti*-orientation, as shown in Fig. 3. Assuming the orientation is the same in solution, the functional groups at C_2 and C_4 would likely fit into a cleft that would be the deepest part of the putative cytidine specificity site. The facts that the C_5 could be replaced by N, as in azacytidine, and the H on C_5 by a Br with relatively small effects on the binding (Table 1) are consistent with the interpretation that the H-bonding in the cleft is the major region of interaction with the pyrimidine base.

Binding of the nucleoside clearly involves contacts with the sugar ring as well. This was indicated in a gross sense by the fact that an analog with cytosine attached in the wrong anomeric conformation had no inhibitory activity (α-cytidine). However, even with analogs that have the normal β-anomeric conformation, binding inhibition was sensitive to the nature and orientation of groups about the C_2 of the ribose ring. The finding that 2′-deoxycytidine was a more potent inhibitor than was cytidine implies that the 2′-OH group is not essential and may even be a partial hindrance. This apparently higher affinity for deoxycytidine than for cytidine is interesting because with polynucleotides rho does not show a preference for DNA over RNA; it binds as well to poly(rC) as to poly(dC) (17) and may even have a slight preference for poly(rC) (10). This lack of DNA preference at the polynucleotide level may be reflecting the contributions of other parts of the polynucleotide binding site on rho, besides the cytidine pocket. Rho contains some sequence segments with identities and similarities to consensus sequences in RNA binding proteins (18) and these may comprise those parts of the binding site that are responsible for the RNA preference at the polynucleotide level.

In cytidine, the 2′-OH group has an axial orientation and is on the opposite side of the ribose ring as the pyrimidine base (Figure 3). In arabinosylcytosine, the 2′-OH group is on the same side as the base and also with an axial orientation (19). Since this analog had moderate competitive activity, the site can accommodate the presence of an OH group with the opposite orientation. However, 2′-O-methylcytidine was virtually ineffective as a competitor. In its crystal structure, the orientation of the C_2′-O bond is equatorial (20). Thus, a likely reason for its lack of inhibition is an inability to fit into the site because of steric hindrance. We propose that rho protein makes a close van der Waals contact with the edge of the ribose ring at the C_2′ position. Since poly(2′-O-methylcytidylate) does not compete with poly(C) for binding to rho whereas poly(dC) does (10), the proposed van der Waals contact appears to be a characteristic of the binding specificity with polynucleotides as with nucleosides.

The C-specific site also appears to be characterized by a strict steric relation between the cleft for the heterocyclic aromatic ring and the positioning groups for the ribose ring. This is suggested by the lack of inhibitory activity of adenosine, which, because of its amino group, might be able to bind into the cleft, but, because of the larger size of the purine ring, would not allow simultaneous contact with functional groups on the ribose ring system.

Another unexpected finding was that none of the nucleotide derivatives of cytidine (5′, 3′ or 2′-CMP) were significantly better or worse competitors than was cytidine (Table 1). This was

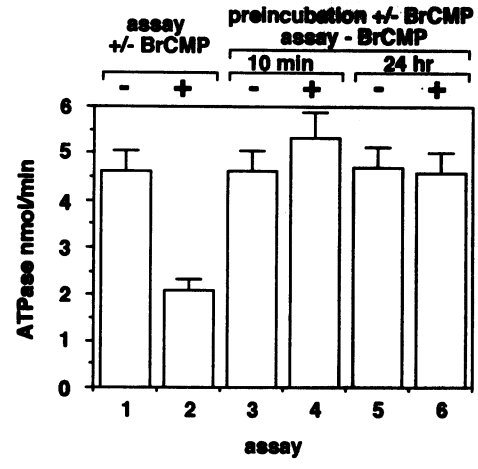

Figure 4. BrCMP inhibits but does not inactivate rho. The assay measures the hydrolysis of ATP with poly(C) as the RNA cofactor, as described in Materials and Methods. For assays +/− BrCMP, 60 ng of rho was assayed either without (−) or with (+) 50 mM BrCMP present. For assays that followed preincubation with +/− BrCMP, rho at 0.3 mg/ml in TKM was first incubated at 23°C either without (−) or with (+) 50 mM BrCMP for the indicated time, then 60 ng was assayed in the standard reaction mixture with no BrCMP added (Note: for the samples preincubated in 50 mM BrCMP the diluted concentration of BrCMP in the assays was 0.5 mM).

unexpected because ionic bonds are important in stabilizing the interaction of rho with mRNA (8), and it is assumed that basic residues comprise some portion of the polynucleotide binding site. This result suggests that these basic residues are not in very close proximity to the cytidine binding pocket. This would also explain why, unlike its interaction with natural mRNA molecules, rho can bind tightly to poly(C) even in 2M KCl (10).

The fact that 3′-CMP was a slightly better inhibitor of binding than was cytidine while 5′-CMP was not suggests that a basic residue may be situated close enough to the side of the ribose binding pocket that accommodates the 3′-phosphate group to make a weak, added bonding interaction. The finding that 2′-CMP was the weakest inhibitor in this group is consistent with the evidence that nucleoside binding was sensitive to other 2′ substitutions. Finally, it is notable that 3′-O-methylcytidine, unlike 2′-O-methylcytidine, was a moderately effective competitor. This result is a further indication that the steric constraints that apply to the 2′ position do not apply to the 3′ position.

Test for possible covalent adduct

One known mechanism for binding of pyrimidine nucleotides to proteins involves the reversible addition of a nucleophilic group to C_6 of the pyrimidine ring (21). A diagnostic property of this kind of reaction is that the addition products with 5-Br pyrimidine analogs often form dead-end products that inactivate the enzyme (22,23). To test whether a nucleophilic addition is involved in the binding of rho to C residues in RNA, we measured the effect of a 5-bromocytidine derivative on rho-ATPase activity. For this study, we used 5-bromocytidine 5′-monophosphate (BrCMP), which binds tightly enough to rho to inhibit ATPase with poly(C). When BrCMP was present at 50 mM during the assay, rho ATPase was inhibited by 50% (Figure 4). However, when it was present at that concentration during preincubation with 0.3 mg/ml

rho and diluted 100-fold prior to performing the ATPase assays, full activity was recovered throughout the range of preincubation times tested (Figure 4). Thus, even though BrCMP evidently forms a Michaelis complex with rho, it does not readily form a dead-end reaction product. In this respect, rho is unlike several other enzymes that are known to bind to pyrimidine nucleotides.

The possibility that formation of a nucleophilic adduct might be a critical step in rho action was first raised because wild type rho has a single cysteine and is sensitive to reagents that react readily with cysteine residues. However, Dombroski and Platt (24) showed that a mutant derivative of rho with the cysteine residue replaced by an alanine was fully active, and Seifried *et al.* (25) showed that the cysteine in wild-type rho could be alkylated without significantly changing rho's functional properties. Thus, the cysteine residue is not essential for rho action. Our results extend those findings because inactivation with BrCMP might have then pointed to the involvement of some other nucleophilic group. Although the possibility that some other residue in rho forms an adduct with cytidine has not been formally eliminated, that hypothesis can now be considered unlikely because of the lack of formation of the diagnostic dead-end reaction product with BrCMP.

CONCLUSIONS

Rho factor contains a binding site for polynucleotides that is strongly specific for single-stranded RNA or DNA containing unpaired cytidylate residues. We show here that much of the specificity of the binding of RNA to this primary, ATP-independent binding site in rho is determined by a domain that can interact with cytidine. This domain apparently consists of a cleft for the pyrimidine ring which can discriminate cytidine by the functional groups that project away from the ribose ring in the *anti* orientation, particularly the presence of an H-bond donor group attached to C_2. This cleft would be in a pocket for the ribose ring that would make a close van der Waals contact with the edge of the ribose ring near C_2'.

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