Model studies have identified 16 conserved positively charged amino acids that form a positive strip pointing toward the center hole of Rho. Fourteen residues were individually changed to either an alanine or a glycine and one to a glutamate. Residues Arg269, Arg272, Lys283, Arg296, Lys298, and Arg299 form a subdomain (locus) located N-terminal to (above) the ATP hydrolysis domain (P-loop) and mutations in these residues led to either inactive Rho or to proteins displaying decreased kcat for poly(C)-dependent ATP hydrolysis, increased Km for ribo(C)10 activation, and decreased transcription termination efficiencies (57–77%) compared with wild-type Rho. Residues Arg247, Lys248, Lys252, and Arg253 form a subdomain (locus) C-terminal to (below) the ATP hydrolysis domain, and mutations in these residues also show a decreased kcat for poly(C)-dependent ATP hydrolysis, an increased Km for ribo(C)10 activation, and a 50–70% decrease in transcription termination, compared with wild-type Rho. Residues Arg312 and Lys336 surround the ATP hydrolysis domain, and mutations in these residues also altered the kinetic properties of Rho. We conclude that the secondary RNA-tracking site consists of amino acids whose putative orientation faces the central hole in Rho and in part reside in two clusters of positively charged residues located above and below the ATP hydrolysis domain.

Rho transcription termination factor is one of several nucleic acid-binding proteins belonging to a family of helicases with a homohexameric structure shaped like a toroid ring that utilizes the hydrolysis of ATP to move along the nucleic acid (1, 2). Rho protein binds to a poorly defined cytosine-rich sequence on nascent RNA called a rut site (Rho utilization), which contains little or no secondary structure. Rut sites are usually found upstream from the start of specific genes or between genes in operons (3), and once Rho binds to the rut site, it tracks 5′ to 3′ toward the stalled RNA polymerase. In a poorly defined mechanism that may require helicase activity (4), Rho disrupts the polymerase-transcript complex, thereby terminating transcription. Bicyclomycin, a commercial antibiotic, has been shown to inhibit Rho function (5) specifically by interfering with the tracking of Rho (6). Bicyclomycin inhibits the growth of many Gram-negative organisms, including Escherichia coli, Shigella, and Salmonella (7) and at least one Gram-positive bacterium, Micrococcus luteus (8), inferring the vital nature of Rho for cellular function (9–11).

The Rho monomer consists of 419 amino acid residues divided into two structural domains, a primary RNA-binding domain (residues 1–151) and the ATP hydrolysis domain (residues 167–419), which is based upon structural similarity with F1-ATP synthase (12). The RNA-binding domain contains a DGGFLR (amino acid residues 60–66) conserved RPN-1 RNA recognition motif (13, 14). A solution structure of the N-terminal 130 residues of Rho has been solved using NMR techniques (15) and a crystal structure is also available (16). The primary RNA-binding domain sits as a cap on the toroid ring, distinct from the ATP hydrolysis domain, and binds RNA tightly. ATPase activity is induced by the addition of poly(C) to Rho. Substitution of poly(dC) for poly(C) leads to tight Rho binding but no ATP hydrolysis. The addition of short oligoribonucleotides (7–10 residues long with a predominance of cytosine residues) to the Rho-poly(dC) complex activates ATPase activity. The activation by ribo(C)10 is thought to occur at the secondary RNA-binding/tracking site (3).

Recently, the projected Q-loop region, positioned on top, inside the toroid ring, facing the central hole of Rho, has been implicated in RNA binding, and this RNA-binding domain changes conformation upon binding ATP (17). A model for Rho tracking coupled to ATP hydrolysis that relies on the structural similarity between Rho and the β-subunit of F1-ATP synthase and utilizes residues facing inside of the central hole in Rho has been put forth (18). A strip of positively charged amino acids on Rho positioned toward the inside of the hole was identified based on the structure of bovine F1-ATP synthase (12, 19) threaded with E. coli Rho sequence and energy minimized (18). This model takes into account three active ATP hydrolysis sites per hexamer, sequential hydrolysis of ATP, Rho activation by short oligoribonucleotides, and the discrimination between hydrolytic and inactive subunits. The strip of positive charges was projected to bind the polyphosphate backbone of the RNA as Rho translocates toward the RNA polymerase. The positive charges were clustered into two separate loci found near the N and C termini surrounding the ATP hydrolysis domain. In this paper, we report the effects of site mutations carried out on these amino acid residues. We document the importance of positive charges for Rho to hydrolyze ATP, to bind RNA, and to terminate transcripts.

**EXPERIMENTAL PROCEDURES**

*Materials and Enzymes—Bicyclomycin was kindly provided by Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan) and was further purified by three successive silica gel chromatographies using 20% methanol-chloroform as the eluant (20). Oligonucleotide primers were synthesized*
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by Genosys Biotechnologies, Inc. (The Woodlands, TX). T4 poly nucleotide kinase, T4 DNA ligase, and restriction enzymes were purchased from Promega Co. (Madison, WI). Pfu DNA polymerase was obtained from Stratagene (La Jolla, CA). The metal chelating column was from Amersham Biosciences.

Radianucleotides ([γ-32P]ATP and [α-32P]CTP (3000 Ci/mmol) were purchased from PerkinElmer Life Sciences, and nucleotides and RNase inhibitors were from Ambion, Inc. (Austin, TX). Polyethyleneimine thin-layer chromatography plates used for ATPase assays were purchased from J.T. Baker, Inc. Riboc(10) was obtained from Oligos Etc. (Wilsonville, OR). All other chemicals were reagent grade.

Expression of Mutant Rho Proteins—One of four expression systems were used to generate soluble and functional mutant Rho proteins: the pET14b expression vector, using host strain BL21(DE3)pLysS (Novagen), the pET14b vector in the salt-induced T7 polymerase host strain HMS174, or BL21SI, the original pET14b vector was used. Expression of mutant Rho using the arabinose promoter in the BL21(DE3)pLysS, HMS174, or BL21SI, the original pET14b vector was used. Expression of mutant Rho using the arabinose promoter in the BL21SI (Invitrogen), the pBAD33 vector (arabinose inducible) (Novagen), the pET14b vector in the salt-induced T7 polymerase host strain (Stratagene). The resulting PCR-amplified plasmid DNA was digested with DpnI and transformed into host strain JM109. Isolated plasmid DNA from the transformed cells was sequenced with an Applied Biosystems 377 sequencer using the Big Dye reaction kit to identify sequences with specific site changes. The entire rbo gene was sequenced to ensure that no other mutations were present in the singly mutated gene.

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mutations, when introduced into the BL21 strains, generated few, if any, transformants and those that grew produced truncated protein although the plasmid was stable in host JM109 (data not shown).

Expression from the pBAD3 vector under the arabinose promoter was generally under tighter control (21). The XbaI–HindIII fragment containing the entire rho gene from the pET14b was placed in pBAD33, transformed into host MG1655, and expression was induced by adding 0.2% arabinose. Expression of K352A, K298A, and R299A were obtained using the pBAD33 vector. The remaining mutants either did not grow when pBAD33 was transformed into MG1655 or did not express protein. Transforming vectors into the MG1655, and expression was induced by adding 0.2% arabinose and 200 mg/ml ampicillin to 0.6–1.0 A600. Expression was induced by the addition of 2–4 x 10⁸ plaque-forming units ml⁻¹ phage CE6 containing the gene for the T7 RNA polymerase, and inducing expression with λ phage CE6 accomplished expression of the remaining mutant proteins from pET14b vectors R160A, R238A, R269A, R272A, K283E, R296A, R353A, and R384A. Individual colonies were grown in LB media containing 0.2% maltose and ampicillin to 0.6–1.0 A600. Expression was induced by the addition of 2–4 x 10⁸ plaque-forming units ml⁻¹ phage CE6 containing the gene for the T7 RNA polymerase. Three h after infection, cells were harvested, lysed, and Rho-purified using the HiTrap metal chelating column bound with Ni²⁺. These mutants overexpressed protein; however, R160A, R269A, and K283E exhibited no poly(C)-dependent ATPase activity and R272A and R384A produced inclusion bodies that could not be solubilized.

Poly(C)-dependent ATP Hydrolysis Activity—The poly(C)-dependent ATPase activity for the Rho mutants was measured as a function of ATP concentrations (Table II). All of the mutant proteins exhibited a decrease in kcat for ATP hydrolysis when compared with wild-type. Wild-type kcat was 2380 min⁻¹, and only R296A approached this value at 2000 min⁻¹. The other mutants showed kcat values one-half or less of wild-type. Three mutants (R160A, R269A, and K283E) were partially soluble but had no ATP hydrolysis activity. The Km for His-tagged wild-type was ~5-fold higher than we reported for wild-type Rho. A large increase in the Km(A) values compared with the wild-type value of 850 min⁻¹ (Table II), and the decrease in the kcat value for ribo(C)₁₀, activation generally mirrored the kcat values determined in the poly(C)-dependent ATPase assay. Of significance, R296A, K348A, and K352A showed kcat values similar with wild-type. The Km(rib(C)₁₀) values for wild-type Rho was 9.0 μM. The K363A mutation showed no detectable poly(dC)-ribo(C)₁₀-stimulated ATP hydrolysis activity, and the poly(C)-dependent ATPase activity was diminished nearly 18-fold. This was the only mutation with a large increase in the Km(ribo(C)₁₀), suggesting that Lys336 has a direct role in ATP binding. This finding supports the notion that residues from neighboring subunits can influence ATP binding across the subunit interface (31).

By comparison, most of the active mutants exhibited Km(rib(C)₁₀) values 5–100-fold greater than wild-type Rho, with the only exception being R347G, which had a Km(rib(C)₁₀) value of 5.6 μM. R296A and K352A stand out as unusual, the kcat for poly(dC)-ribo(C)₁₀-dependent ATP hydrolysis exceeded that of wild-type yet the Km for ribo(C)₁₀ was 500 and 1000 μM, respectively, which was 50–100-fold greater than wild-type Rho. A large increase in the Km(rib(C)₁₀) coupled with a slight increase in kcat for ATP hydrolysis suggest that the on-rate for ribo(C)₁₀ was lower. To support this notion transcription termination efficiencies should be considerably less for these mutants than for wild-type.

Transcription Termination—In vitro transcription termination reactions were measured using the modified trp operon fragment (22), as reported (6). Fig. 1A shows the transcription termination reactions of wild-type Rho, R212G, R296A, K283A, and R299A, which are located at the putative N-terminal locus of the RNA tracking site. Lane 12 shows the run-off transcript (labeled A in Fig. 1A) in the absence of Rho; lane 1 shows the transcripts in the absence of Rho but with the inclusion of 28
μM rifampicin, lane 2 shows transcript produced in the presence of wild-type Rho, and lane 3 shows the effect of 50 μM bicyclomycin on wild-type Rho. Transcription termination efficiencies as a percentage of wild-type Rho for the mutant proteins as depicted in Fig. 1A and B, are summarized in Table II. Mutant R212G generated only 14% of the terminated transcripts compared with wild-type Rho, whereas mutants R296A, K298A, and R299A generated 53, 32, and 23% terminated transcripts, respectively. The terminated transcripts for the mutants were longer than those seen for wild-type Rho. The terminated transcripts are labeled 1, 2, and 3 in Fig. 1A. Wild-type Rho produced terminated transcripts dominated by 2 and 3 with a small amount of 1. The mutant Rho proteins predominately produced the longer terminated transcripts 1 and 2 but little, if any, 3, the shortest terminated transcript. The production of longer terminated transcripts was reminiscent of the intermediate-size transcripts seen in the titration of Rho with the antibiotic bicyclomycin (6) and the transcripts generated from bicyclomycin-resistant Rho mutants (6). We previously concluded that bicyclomycin slowed the 5′ to 3′ translocation of Rho down the RNA, allowing the RNA polymerase to proceed further along the DNA before transcription termination. A similar argument can be made for the effects of amino acid substitution on transcription termination.

Fig. 1B shows transcription termination from mutations (R347G, K348A, K352A, and R353A) clustered on the C-terminal RNA tracking locus. Mutants R347G, K348A, and K352A generated 50% or less terminated transcripts than wild-type, and the terminated transcripts were longer. The mutant R353A showed 97% transcription termination efficiency and gave predominantly larger terminated transcripts than seen for wild-type Rho. This mutant was of interest because the \( k_{cat} \) for ATP hydrolysis was between one-third and one-fourth that of wild-type but transcription termination efficiency was not affected. The rate of ATP hydrolysis did not correlate well with transcription termination efficiency. We found a better correlation between the transcription efficiency and the \( K_m(\text{Rib}(\text{C})_{10}) \) value in the poly(dC)-riboc\( (\text{C})_{10} \) assay. Bicyclomycin effectively inhibited mutant Rho proteins as seen in Fig. 1. Also, the kinetics of Rho inhibition by bicyclomycin was measured as a function of ATP concentrations using the poly(C)-dependent ATPase assay and we observed that the \( K_m \) for bicyclomycin was not altered (data not shown).

RNA Binding Experiments—The binding of trp t' RNA to Rho mutants was measured to determine whether amino acid substitutions at putative secondary RNA-binding/tracking sites affected RNA binding at the primary site. Fig. 2 shows the binding curves and the percent Rho binding RNA for several mutants and the data is summarized in Table III. The filter binding efficiency (23) for wild-type Rho was determined to be very close to unity. Because of the complexity of the binding, the data was analyzed by three separate methods. The simplest was the L50 value, which was the concentration of Rho that gave half-maximal retention of trp t' RNA. Several mutants showed sigmoidal behavior upon RNA binding, and the Hill constants for sigmoidal binding (determined from the slope of the Hill plot at the corresponding L50 value) varied between 1.9 and 4.4 similar to that reported (28). The \( K_m \) values were calculated using Equation 2 from Ref. 24 and the Hill constants were as described in Table III. Finally, the \( n \)th root of \( K_m \) was determined. Wild-type Rho showed the tightest binding with a L50 of binding occurring at 0.81 ± 0.03 mM, whereas the weakest binding mutant, K298A, showed a L50 of >9.0 mM. The L50 values for the other mutants fell within a range of 1.8 to 3.6 mM. Binding of trp t' to R296A, K298A, R353A, and K348A showed binding efficiencies near 50%. Data presented in Table III suggested that mutations distant from the known primary binding site influence tight RNA binding only slightly but may have weakened the hexamer formation in the absence of RNA.

Fluorescence—Fig. 3 shows the fluorescence emission spectra of F355W and wild-type Rho in the absence and presence of 100 μM ATP. We observed that in proceeding from wild-type to F355W, the emission maximum shifted from 375 to 350 nm and increased in emission intensity in the absence of ATP. This emission intensity increase was because of the additional tryptophan residue in the F355W mutant, an increase from one to two tryptophan residues. The blue shift suggests that the additional tryptophan residue was in a solvent inaccessible location in Rho consistent with its predicted placement. The addition of 100 μM ATP caused a 74% decrease in the fluorescence intensity of F355W at 350 nm. By comparison, less than 10% of the fluorescence intensity of wild-type Rho was quenched at 350 nm upon ATP (100 μM) addition. These data provided strong evidence that Phe\( ^{355} \) is close to the adenosine-binding site, in agreement with the generated structural model for Rho based on the crystal structure of F1-ATP synthase (32). The relative amount of fluorescence quenching upon ATP addition was greater than that seen for F1-ATP synthase, presumably because F355W Rho has only one other tryptophan residue.

### DISCUSSION

The secondary RNA-binding/tracking site is poorly defined, and only a few mutations with phenotypes consistent with defects in tracking have been identified. Mutations (K352E (30), M327T (33), and E342G (33)) altering ribo(C)\( _{10} \) binding to Rho have been isolated. Many mutants have decreased \( k_{cat} \) for ATP as measured by poly(C)-stimulated ATP hydrolysis. The positions of these mutations are distant from the ATP hydrolysis pocket and are scattered on the inside face of the hexamer (33). We have proposed a structural model of Rho based on the crystal structure of F1-ATP synthase (18) where the secondary tracking site lies within the central hole, and have provided
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Fig. 1. A, transcription termination mediated by Rho mutants clustered in the N-terminal locus of the proposed tracking site. Lane 1, reaction carried out in the presence of 28 μM rifampicin without Rho or bicyclomycin; lane 2, transcription termination carried out in the presence of 70 nM Rho hexamer; lane 3, transcription termination carried out in the presence of 70 nM Rho hexamer in the presence of 50 μM bicyclomycin. Lanes 4, 6, 8, and 10, transcription termination carried out in the presence of 70 nM R212G, R296A, K298A, and R299A mutant Rho hexamers, respectively, without bicyclomycin, and lanes 5, 7, 9, and 11, transcription termination carried out in the presence of 70 nM R212G, R296A, K298A, and R299A mutant Rho hexamers, respectively, in the presence of 50 μM bicyclomycin. Lane 12, transcription termination reaction in the absence of Rho without rifampicin and bicyclomycin. The transcript labeled A is the run-off transcript; transcripts 1, 2, and 3 are progressively shorter terminated transcripts. The more efficient Rho is the shorter the transcripts. B, transcription termination mediated by Rho mutants clustered in the C-terminal locus of the proposed tracking site. Lane 1, transcription termination carried out in the presence of 70 nM Rho hexamer; lane 2, transcription termination carried out in the presence of 70 nM Rho hexamer in the presence of 50 μM bicyclomycin. Lanes 3, 5, 7, and 9, transcription termination carried out in the presence of 70 nM Rho hexamer in the presence of 50 μM bicyclomycin. Lanes 4, 6, 8, and 10, transcription termination carried out in the presence of 70 nM R347G, K348A, K352A, and R353A mutant Rho hexamers, respectively, without bicyclomycin, and lanes 5, 7, 9, and 11, transcription termination carried out in the presence of 70 nM R347G, K348A, K352A, and R353A mutant Rho hexamers, respectively, in the presence of 50 μM bicyclomycin. The transcript labeled A is the run-off transcript, whereas transcripts 1, 2, and 3 are progressively shorter terminated transcripts. Lane 11, transcription termination reaction in the absence of Rho and bicyclomycin.

Fig. 2. Trp t RNA binding to mutant Rho proteins. Binding of trp t RNA at the primary RNA recognition site was compared among the mutant Rho proteins with mutations affecting RNA binding at the RNA-tracking site. All samples contained 1.2 nM 32P-labeled trp t RNA and the indicated concentrations of Rho (hexamer) in the absence of adenosine nucleotides, wild-type Rho (●), R296A (○), K298A (▲), K352A (■), R353A (■), F395W (□), R212G (●), R347G (○), and K348A (▲).

Enzymatic Activities—Three enzymatic activities helped characterize the mutant and wild-type Rho proteins. Poly(C)-dependent ATP hydrolysis measured the ability of poly(C) to activate Rho-dependent ATP hydrolysis. Poly(C) binds strongly to both the primary and secondary binding sites providing maximum ATP hydrolysis rates. In this assay, the ATP concentrations were varied, whereas poly(C) was maintained at saturating levels. The $K_{m}$ value was usually insensitive to parameters affecting RNA binding, whereas the $k_{cat}$ for ATP hydrolysis reflected activation by RNA binding at the tracking site. Because poly(C) binds tightly to both primary and secondary binding sites, this assay is not an effective measure of Rho-RNA interactions necessary for Rho translocation. RNA binding interactions at the tracking site can be determined by measuring the poly(dC)-ribo(C)10-dependent ATP hydrolysis activity as a function of ribo(C)10 concentration. Both $k_{cat}$ for ATP hydrolysis and $K_{m}$ (ribo(C)10) are indicators of altered RNA binding at the tracking site. Transcription termination is the most sensitive indicator of overall Rho function, exceeding ATP hydrolysis activity. For example, the inhibitor, bicyclomycin, has an $I_{50}$ value for poly(C)-dependent ATP hydrolysis of 60 μM, whereas the transcription termination $I_{50}$ value for bicyclomycin was 5 μM (19). Anti-termination was achieved by slowing the tracking rate such that Rho cannot catch the RNA polymerase, and under these conditions ATP hydrolysis may only be marginally affected. This kinetic coupling model suggests that small changes in ATP hydrolysis may not be responsible for large increases in anti-termination.

Trp t RNA Binding—The trp t RNA binding measured whether the selected mutations adversely altered recognition at the rut (primary binding) site contributing to the dominant-negative behavior during mutant protein overexpression. The dominant-negative effect can be reasoned as mutant Rho competing with background wild-type for rut sequences. The L50 or nth root of $K_{m}$ for trp t binding was not significantly altered by these mutations (Table III). This data suggests that rut site recognition was not an important factor in the phenotype of the
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TABLE III

Trp t′ RNA binding to mutant Rho proteins

| Rho       | L50(nMhex) | % Rho binding | Hill constant | Kcat(nMhex) | Kd(nMhex) |
|-----------|------------|---------------|---------------|-------------|------------|
| Wild-type | 0.81 ± 0.03| 100           | 1.21 ± 0.14   | 1.20 ± 0.15 |
| R296A     | 3.56 ± 0.24| 50            | 5.49 ± 0.31   | 2.57 ± 0.34 |
| R298A     | 9.11 ± 0.13| 46            | 81.12 ± 6.17  | 5.12 ± 0.07 |
| R325A     | 1.83 ± 0.01| 90            | 5.92 ± 0.1    | 2.09 ± 0.14 |
| R353A     | 2.94 ± 0.16| 44            | 5.50 ± 1.2    | 1.52 ± 0.12 |
| R212G     | 3.44 ± 0.26| 90            | 48.7 ± 13.8   | 4.46 ± 0.17 |
| R347G     | 2.46 ± 0.30| 60            | 13.79 ± 4.2   | 1.84 ± 0.23 |
| K348A     | 1.86 ± 0.01| 44            | 1.24 ± 0.02   | 1.07 ± 0.02 |
| F355W     | 1.93 ± 0.11| 87            | 7.94 ± 0.65   | 2.02 ± 0.08 |

*Kd* is the nth root of *Kcat*.

FIG. 3. Fluorescence emission spectra of wild-type and F355W Rho proteins. Rho concentrations were 100 nM, and the spectra were recorded using an excitation wavelength of 280 nm in the absence and presence of 100 μM ATP.

mutation. These binding experiments were done in the absence of adenosine nucleotides, which are reported to increase RNA binding affinity to Rho (24). However, decreased total RNA binding (percent Rho binding RNA) was seen for several mutants raising the possibility that hexamer formation was weakened in the absence of RNA. R296A showed 50% less trp t′ RNA binding, however, this mutant also showed a higher *kcat* for poly(C)-dependent ATP hydrolysis than wild-type. This suggests that hydrolysis activity was insensitive to small differences in the L50 for trp t′ binding. Whereas trp t′ binding was a measure of the primary RNA binding affinity, the *Km* for ribo(C)10 was a better measure of RNA binding to the secondary site. Most of the mutants were defective in transcription termination and displayed large increases in the *Km* for ribo(C)10. These findings suggested that the mutations were acting at the secondary RNA-binding site primarily and the dominant negative phenotype arose from the slower tracking rates (poorer RNA binding at the secondary RNA-binding site) in the mutant Rho proteins rather than the initial binding step of mutant Rho protein to the rut sequences. Slower tracking results in decreased termination and ultimately in cell death.

The Model—Fig. 4 shows a dimeric model of Rho based upon the crystal structure of an α and β subunit pair of F1-ATP synthase (29) threaded to the Rho sequence and energy minimized as described (18). This figure shows only residues 151–419 including the ATP binding and hydrolysis pocket; the primary RNA recognition domain, residues 1–150, has been removed. Outlined in black boxes are two putative secondary RNA (tracking) loci facing the central hole or Rho; positively charged residues altered by site mutations are identified in blue and placed in a rectangle box. The green subunit is depicted as binding ATP (red), whereas the yellow strand is depicted as not binding ATP.

next to the N-terminal RNA recognition domain, residues 151–340. The ATP hydrolysis pocket is part of this subdomain including the P-loop residues 181–187 (12) and includes the bicyclomycin binding pocket defined by mutations affecting antibiotic binding (35), residues 219, 266 (5), 210 (8), 208, and 337 (18). The C-terminal or bottom locus, residues 341–419, was located below the ATP hydrolysis domain and contains the adenosine-binding pocket (Fig. 5). Both loci contain positively charged residues, which when altered influence the RNA tracking ability of Rho. The fluorescence quenching of tryptophan emission by ATP (Fig. 3) supports this structural arrangement and our assignment of the ATP-binding domain.

The Top or N-terminal Locus—Residues Arg212, Arg238, Arg269, Arg272, Lys283, Arg286, Arg289, and Lys336 are found in the N-terminal locus pointing toward the central hole of Rho. Mutations in three of these residues resulted in inactive Rho: R272A formed insoluble inclusion bodies; R269A and K283E were isolated, purified, and shown to have low poly(C)-stimulated ATP hydrolysis activity. Lysine 283 on the Q-loop was also shown to be important in Rho ATP hydrolysis activity (36). R238A, R296A, K298A, and R299A displayed dominant-negative growth suggesting competition with the host wild-type Rho but preventing effective termination. We found that most of the mutant proteins had altered kinetic properties (*Km*(ATP) and *kcat*) in the poly(C)-dependent ATPase assay com-
Based on the structure of F$_1$-ATP synthase, ATP hydrolysis, increased $K_m$ on the other face, presumably binding RNA.

The Bottom or C-terminal Locus—Residues R347G, K348A, K352A, R353A, and R384A in the C-terminal locus, speculated to be involved in Rho-RNA tracking, were selected for study. R347G, K348A, K352A, and R353A are believed to reside in the putative H-helix, which on one face is projected to bind to the adenosine ring of ATP and on the other face to RNA. Fig. 5 shows the positions of these residues in relation to the ATP binding. Two other residues, Lys$^{402}$ and Lys$^{407}$, were considered promising but were not pursued. Completing our mutational studies for this locus was F355W, which positioned a cryptophan residue near the ATP binding pocket.

R384A and R160A were only expressed as inactive inclusion bodies. Moreover, these inclusion bodies were only generated in the phage-induced expression system, and transformed cells carrying this mutant in the other expression systems failed to grow. The observed dominant-negative phenotype for these mutants suggested a role in RNA tracking.

Mutants R347G, K348A, K352A, and R353A fell into two types, those with $K_m$(ribo(C)$_{10}$) values similar to wild-type Rho and those with $K_m$(ribo(C)$_{10}$) values 20–100 times higher. R347G and R353A had similar $K_m$(ribo(C)$_{10}$) values to wild-type, but their $K_m$(ATP)$_{10}$ values were lower than those of native enzyme. The $k_{cat}$ for ATP hydrolysis for R347G and R353A was 9 and 25% of wild-type in the poly(C)-dependent ATPase assay and 19 and 37% for the poly(dC)-ribo(C)$_{10}$-mediated ATPase assay. Transcription termination for R347G was only 41% of wild-type Rho and produced only the largest terminated transcript, whereas R353A activity was almost identical to wild-type Rho and showed only minor differences in the termination pattern (Fig. 1B). R353A showed a 3.6-fold increase in the L50 for trp RNA binding and only 44% of this Rho bound RNA, but this mutant still effectively terminated transcription and did not have an altered $K_m$ for ribo(C)$_{10}$.

Mutants K348A and K352A showed increased $K_m$(ribo(C)$_{10}$) values with wild-type Rho ($K_m$(ribo(C)$_{10}$) = 9 $\mu$M) at 286 and >1000 $\mu$M, respectively. K348A showed a defective $k_{cat}$ for ATP hydrolysis, a decreased $K_m$(ATP)$_{10}$ value, and a transcription termination efficiency that was 30% of wild-type. Again, there was a strong correlation between $K_m$(ribo(C)$_{10}$) and transcription termination efficiency. We found that the $k_{cat}$ for poly(dC)-ribo(C)$_{10}$-dependent ATP hydrolysis was greater than wild-type Rho and that this mutant displayed an overall pattern of inhibition comparable with R296A, showing decreased transcription termination efficiency but increased ATP hydrolysis, which suggests that K352A behaves as an uncoupler.

The E. coli F$_1$-ATP synthase $\beta$ subunit counterpart of Phe$^{355}$, $\beta$Tyr$^{331}$ (28), is base stacked with the adenosine and the tryptophan mutant Y331W shows fluorescence quenching upon ATP binding (32, 28). The Rho F355W mutation decreased the $k_{cat}$ for poly(C)-dependent ATP hydrolysis by only 50%, whereas $K_m$(ATP)$_{10}$ increased from 57 to 83 $\mu$M. Furthermore, transcription termination was not affected by this amino acid substitution. Fig. 3 shows a blue shift in fluorescence emission maximum from 380 to 350 nm and an increase in total fluorescence compared with wild-type Rho. A large decrease in fluorescence was observed for the F355W mutant upon the addition of 100 $\mu$M ATP but not for wild-type. These data suggested that Rho Phe$^{355}$, like F$_1$-ATP synthase $\beta$Tyr$^{331}$, interacts strongly with ATP.

Fig. 5 shows the orientation of amino acids Arg$^{347}$, Lys$^{348}$.
Mutations in the Rho Transcription Termination Factor

Lys\textsuperscript{352}, Arg\textsuperscript{353}, and Phe\textsuperscript{355} with respect to ATP binding. Lys\textsuperscript{348} and Lys\textsuperscript{352} point out of the opposite face of the helix from Phe\textsuperscript{355}, which binds ATP. Losses of positive charges at these residues caused a large increase in the \(K_m\) of ATP for Lys\textsuperscript{348} and Phe\textsuperscript{355} smaller than wild-type Rho, suggesting that RNA binding at the C-terminal locus may alter ATP binding. Mutations introduced at Arg\textsuperscript{347} and Arg\textsuperscript{353} did not greatly affect the RNA binding at the tracking site. We found that the \(K_m\) for ATP, for Lys\textsuperscript{348} was smaller than wild-type Rho, suggesting that RNA binding at the C-terminal locus may alter ATP binding. Mutations introduced at Arg\textsuperscript{347} and Arg\textsuperscript{353} did not greatly affect the L50 for trp tRNA binding and are positioned away from the putative RNA binding face of the H-helix. Arg\textsuperscript{347} is close to the \(\beta\) phosphate of ATP, consistent with the large decreases in \(K_m\) for ATP hydrolysis observed for R347G in the poly(C)-dependent and the poly(dC)-ribo(C)\textsubscript{10}-dependent ATPase assays.

Our model (18) involves alternate binding and release of RNA at two loci in Rho catalyzed by ATP binding, hydrolysis, and ADP release in a sequential mechanism and our data, on the effect site mutations on RNA binding to the tracking site is consistent with this model. Studies are in progress that measure ATP binding to Rho using F355W fluorescence quenching under different ligand binding conditions and the use of double mutants geared to detect alternating RNA and ATP binding.

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