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Interaction of thiostrepton with an RNA fragment derived from the plastid-encoded ribosomal RNA of the malaria parasite

M. JOHN ROGERS,[1] YURY V. BUKHMAN,[2] THOMAS F. McCUTCHAN,[1] and DAVID E. DRAPER[2]

[1]Growth and Development Section, Laboratory of Parasitic Diseases, National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892-0425, USA
[2]Department of Chemistry, Johns Hopkins University, Baltimore, Maryland 21218, USA

ABSTRACT

Although eukaryotes are not generally sensitive to thiostrepton, growth of the human malaria parasite *Plasmodium falciparum* is severely inhibited by the drug. The proposed target in *P. falciparum* is the ribosome of the plastid-like organelle (35 kb circular genome) of unknown function. Positive identification of the drug target would confirm that the organelle is essential for blood-stage development of *Plasmodium* and help clarify the plastid's biological role. The action of thiostrepton as an antibiotic relates to its affinity for a conserved domain of eubacterial rRNA. Its effect on organelles is unknown. Because a number of different point mutations within the *Escherichia coli* domain abrogates thiostrepton binding, extensive sequence differences between eubacterial and plastid domains brings into question the site of drug action. We have examined temperature-dependent hyperchromicity profiles of synthetic RNAs corresponding to domains in the plastid and cytoplasmic RNAs of *P. falciparum*. Thiostrepton induces a tertiary structure in the plastid-like fragment similar to that seen in eubacterial rRNA, even though the two share only about 60% sequence identity. A single point mutation in the plastid-like fragment removes thiostrepton-dependent tertiary structure formation. Thus, the plastid and eubacterial RNAs share a stabilized tertiary structure induced by the drug. This direct indicator of drug sensitivity in eubacteria suggests that the plastid-encoded ribosome is similarly sensitive to thiostrepton and that the plastid is the site of drug action. Correlation of thiostrepton-sensitive and -resistant phenotypes with physical parameters suggests thiostrepton resistance as a selectable marker for plastid transformation.

Keywords: antibiotic; *Plasmodium falciparum*; ribosome; RNA hyperchromicity; transcription

INTRODUCTION

Many antibiotics interact directly with functional regions of eubacterial rRNA (Cundliffe, 1990). Several classes of antibiotics can be footprinted chemically to 16S rRNA (Moazed & Noller, 1987a) and 23S rRNA (Moazed & Noller, 1987b), and the binding to the A site of 16S rRNA by aminoglycoside antibiotics has been defined structurally (Fourmy et al., 1996). Many of the details of antibiotic specificity for rRNA are, however, poorly understood. The stabilization of one transient state in a pool of many possible tertiary structures is a known phenomenon that can be critical to the activity of ribonucleoprotein complexes (Weeks & Cech, 1996). In some cases, stabilization of RNA tertiary structure is necessary for catalysis. Alternatively, if the drug interaction stabilizes an RNA structure not found in the absence of drug (Draper et al., 1995), the ribosome may be trapped in an inactive state (Cundliffe, 1990; Brink et al., 1994). Understanding the mode of binding to a transient tertiary structure would be of considerable interest in identifying new RNA drug interactions and for drug design.

We found recently that the antibiotic thiostrepton inhibits the growth of in vitro (blood stage) cultures of the human malaria parasite *Plasmodium falciparum* in the micromolar range, and suggested the site of action to be the rRNA encoded by the plastid-like 35-kb circular genome (McConkey et al., 1997). This genome is contained in an organelle of unknown function and its presence in *P. falciparum* may be a result of secondary endosymbiosis (Wilson et al., 1994; Palmer & Delwiche, 1996). Antibiotics of the thiazolyl class, of which thiostrepton is an example, are highly modified peptides that bind a conserved region of 23S rRNA to

Reprint requests to: Thomas F. McCutchan, Growth and Development Section, Laboratory of Parasitic Diseases, Building 4, Room B1-28, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892-0425, USA; e-mail:mcutchan@helix.nih.gov.

inhibit elongation (Cundliffe, 1990), and mutants resistant to thiostrepton are hypersensitive to inhibitors of the peptidyl transferase site (Mankin et al., 1994). The thiostrepton binding site has been localized to a small, 58-nt fragment, corresponding to nt 1051–1109 in *Escherichia coli* 23S rRNA (Fig. 1), with a conserved secondary structure (Gutell et al., 1992). Part of the structure (nt 1093–1098) forms a stable, hexanucleotide loop (Fountain et al., 1996; Huang et al., 1996). Temperature-dependent hyperchromicity of the *E. coli* 1051–1109 fragment reveals the presence of a tertiary structure that is greatly stabilized by Mg^{2+} and NH_4^+ in preference to other monovalent and divalent cations (Wang et al., 1993; Laing & Draper, 1994; Laing et al., 1994). This unusual specificity for ions is a useful diagnostic test for the presence of tertiary structure. The tertiary structure is, by itself, marginally stable under physiological conditions (melting temperature, T_m, 35–40 °C), although binding of the conserved ribosomal protein L11 stabilizes the structure considerably (Xing & Draper, 1995). Thiostrepton also stabilizes the tertiary structure, converting the lowest temperature unfolding transition to a sharper peak at a somewhat higher temperature (Draper et al., 1995). The thio-

FIGURE 1. Primary and secondary structures of RNA fragments encoded by rDNA from the plastid-like (35-kb circular genome) and the nuclear genome of *P. falciparum*, with the domain from *E. coli* 23S rRNA for comparison. **A:** Primary sequence of the for *E. coli* 23S rRNA (nt 1051–1108) with the plastid-like (35 kb) encoded LSU rRNA and cytoplasmic LSU rRNAs from *P. falciparum*. Arrows indicate changes in the cytoplasmic S-type domains. **B:** Secondary structure of the *E. coli* domain, with shaded regions positions involved in thiostrepton binding. The fragments encoded by the 35-kb plastid-like genome (Gardner et al., 1993), indicated are changes at the first base pair for transcription by T7 RNA polymerase, and at nucleotide corresponding to *E. coli* position 1067 to test thiostrepton binding in the 35R construct. The fragments of the nuclear-encoded A- and S-type genes. The A-type is expressed predominantly in asexual (blood) stages of the parasite (Waters et al., 1995), and the S-type expressed in sporozoites isolated from the salivary gland of infected mosquitoes (Rogers et al., 1996). Changes indicate where positions in the S-type fragment differ from the A-type.

strepton–RNA binding affinity and the T_m of the thiostrepton–RNA complex increases with Mg^{2+} concentration and with the substitution of NH_4^+ for Na^+ ions (Wang et al., 1993; Draper et al., 1995), indicating that the drug-stabilized structure is similar to the tertiary structure found in the free RNA. In this work, we compare melting of the *P. falciparum* RNA fragments derived from nuclear- and plastid-encoded rRNAs under different conditions (with and without Mg^{2+}, NH_4^+ versus Na^+, and with and without thiostrepton). These data confirm the plastid-encoded RNA fragment as the site of thiostrepton binding.

RESULTS

To investigate directly the effect of thiostrepton on the *P. falciparum* rRNAs, the 35-kb encoded rRNA fragment corresponding to the 1051–1109 domain in *E. coli* 23S rRNA was synthesized by transcription with T7 RNA polymerase of a synthetic gene (Fig. 1; see Materials and Methods). Also, a mutant (35R) with an A → U change analogous to position A1067 in *E. coli* rRNA was made in the plastid-encoded rRNA (Fig. 1B). This mutation corresponds to the site of ribose methylation to produce 2′-*O*-methyladenosine in *Streptomyces azureus* (Thompson et al., 1988), the organism that synthesizes thiostrepton, and is potentially a site of interaction of thiostrepton and micrococcin (Rosendahl & Douthwaite, 1994). Micrococcin is a related antibiotic that stimulates rather than inhibits ribosome-dependent GTP hydrolysis (Cundliffe & Thompson, 1981). Additionally, *P. falciparum* has nuclear-encoded rRNAs that are regulated developmentally (Waters et al., 1995; Rogers et al., 1996), with an A-type rRNA expressed predominantly in asexual (blood) stages and an S-type expressed in purified sporozoites isolated from the salivary glands of infected mosquitoes (McCutchan et al., 1995). The fragments corresponding to these rRNAs were also synthesized (Fig. 1B). The rRNAs encoded by the mitochondrial-like genome are fragmented (Feagin et al., 1991) and fragments resembling the conserved 1051–1109 domain in *E. coli* 23S rRNA have yet to be identified (Feagin et al., 1992, 1997). The mitochondrial rRNA fragments were therefore not studied.

Melting profiles (first derivative of UV hyperchromicity as a function of temperature) for the transcript of the plastid-encoded RNA fragment and its mutant (35R) are shown in Figure 2. The plastid-encoded RNA fragment in the absence of thiostrepton shows no evidence of the unusual ion specificity that is diagnostic for the presence of tertiary structure of the *E. coli* homologue (Draper et al., 1995); the melting profiles are relatively insensitive to Mg^{2+} concentration present, and substitution of Na^+ for NH_4^+ results in no significant change (data not shown). This may be due to the higher number of A-U base pairs in the plastid-encoded fragment resulting in a lower intrinsic stability, because the broad, featureless melting of the RNA that begins at temperatures less than 10 °C (Fig. 2A) also suggests that the RNA has not folded into a stable tertiary structure. With addition of thiostrepton, the melting profile changes dramatically; unfolding does not begin until approximately 15 °C, and a new, sharp transition centered at 32 °C appears (Fig. 2A). These changes are more dramatic than seen in the *E. coli* RNA fragment (Draper et al., 1995), and indicate that thiostrepton interacts strongly with the plastid-encoded rRNA domain. When the unfolding experiments are repeated with Na^+ substituted for NH_4^+, the appearance of the melting profile is similar, but the sharp peak has shifted to 22 °C (data not shown). The additional stability of the complex with NH_4^+ supports the

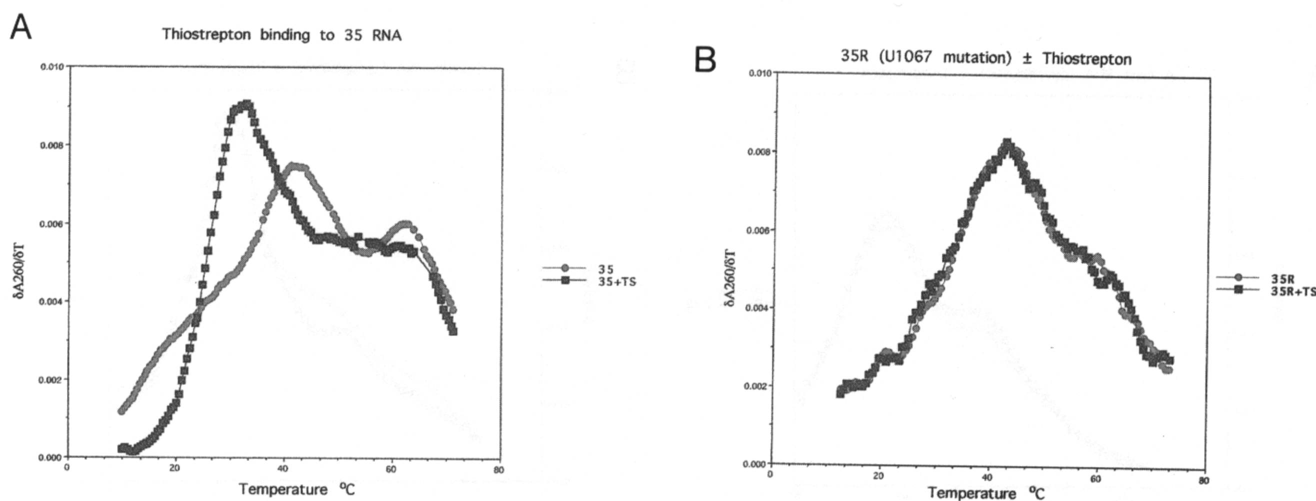

FIGURE 2. Melting profiles of the plastid-encoded (35 kb) fragment detected by changes in absorbance at 260 nm. **A:** Melting profile of the plastid-encoded fragment (35) in the absence (red) and presence (blue) of thiostrepton (TS). **B:** Melting profile of the 35R fragment (A → U mutation) in the absence (red) and presence (blue) of thiostrepton (TS).

suggestion that the RNA has adopted a tertiary structure similar to that seen in the *E. coli* rRNA.

Additional support for similar interactions of thiostrepton with the plastid-encoded fragment and *E. coli* rRNA fragments comes from experiments with the plastid-encoded fragment with the A → U mutation (35R; Fig. 2B). The melting profile of this fragment in the absence of thiostrepton is very similar to that of the 35 RNA fragment, whereas addition of thiostrepton causes no detectable changes (Fig. 2B). Thus, the nucleotide corresponding to A1067 in the *P. falciparum* plastid-encoded fragment is implicated in the formation of the thiostrepton binding site, as in eubacterial rRNAs.

In contrast, neither of the nuclear-encoded fragments shows thiostrepton-induced changes in melting profiles (Fig. 3A,B). Although the A-type fragment shows the Mg^{2+}-dependent tertiary structure seen with the *E. coli* domain (data not shown), the curves in the presence and absence of thiostrepton are virtually superimposable. Interestingly, the melting profile of the S-type fragment has no dependence on Mg^{2+} concentration (data not shown), and the melting profile shows subtle differences from that of the A-type RNA (Fig. 3B). This implicates base pair 1059–1079 and nt 1084 (Fig. 1) in formation of tertiary structure (Y. Bukhman, unpubl. results), although mutation at position 1084 in *E. coli* fragment has a small, about twofold, effect on reducing thiostrepton binding and L11 binding (Ryan & Draper, 1991). However, the melting profiles for the S-type fragment in the presence and absence of thiostrepton are almost superimposable.

DISCUSSION

The mode of action of thiostrepton is probably related to stabilizing a transient, unstable tertiary structure of a domain of rRNA that is required during protein synthesis (Cundliffe, 1990). It is important to correlate interaction of thiostrepton with the plastid-encoded rRNA (McConkey et al., 1997) as the cause of growth inhibition of *P. falciparum* with thiostrepton. Comparison of thiostrepton-dependent tertiary structures using synthetic RNAs derived from plastid- and nuclear-encoded domains as substrates confirms the proposal that the plastid-encoded RNA is the target for thiostrepton in *P. falciparum*. The thiostrepton-stabilized structure is reflected in the transition centered at 32 °C with the domain derived from the rDNA of the plastid genome, and the structure is removed by a single point mutation that corresponds to a thiostrepton-resistant mutation in eubacteria. Because both the nuclear-encoded fragments show no binding to thiostrepton, these data also support the importance of A1067, which is universally conserved in eubacteria and organellar rRNAs (Gutell et al., 1992), in thiostrepton interaction. The corresponding position is a G in the *P. falciparum* nuclear-encoded fragment, as is the case for the rRNAs of eucarya. Although thiostrepton is familiar as an antibiotic, the mode of action of thiostrepton in *Streptomyces* spp. is as an inducer of gene expression (Murakami et al., 1989). In this organism, thiostrepton reacts directly with free cysteines of thiostrepton-induced proteins through the dehydroalanine residues of the antibiotic (Chiu et al., 1996). We have shown structural changes that take place upon addition of thiostrepton to the plastid-encoded fragment, which makes the binding to thiostrepton-induced proteins unlikely as the cause of growth inhibition in *P. falciparum*. Additional support for direct binding of thiostrepton to the plastid-encoded rRNA may be provided by binding assays (Thompson & Cundliffe, 1991) or footprinting assays (Rosendahl & Douthwaite, 1994). However, isolation of sufficient quantities of plastid ribosomes from the parasite remains problematic.

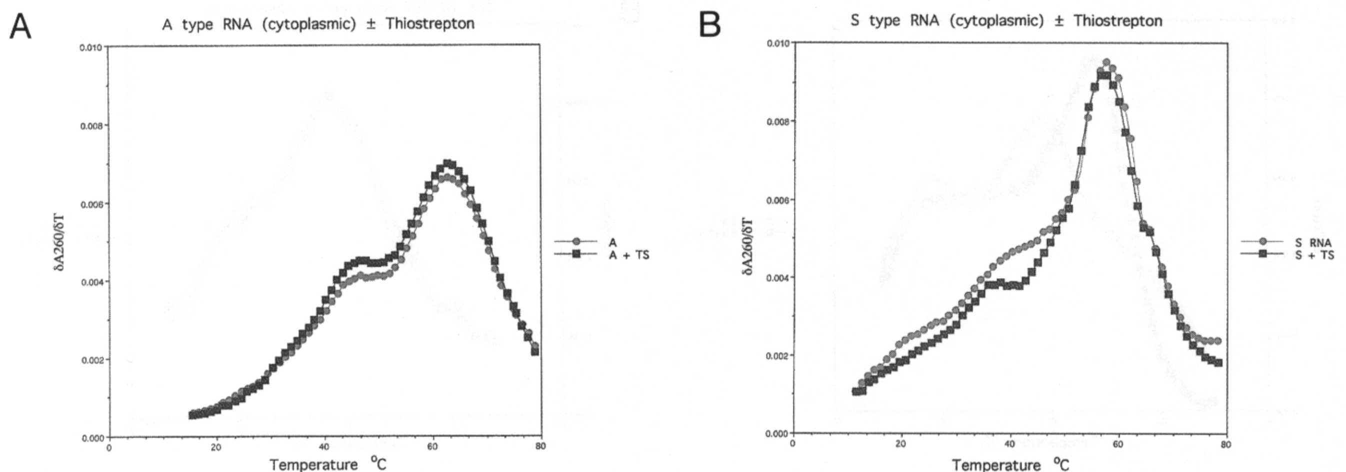

FIGURE 3. Melting profile of the nuclear-encoded A- and S-type fragments. **A:** Melting profile of the A-type fragment (expressed in blood stages) in the absence (red) and presence (blue) of thiostrepton (TS). **B:** Melting profile of the S-type fragment (expressed in mosquito stages) in the absence (red) and presence (blue) of thiostrepton (TS).

Identification of the fragment of the plastid-encoded large subunit rRNA as the target for thiostrepton, distinct to the nuclear-encoded fragments, indicates the potential use of these antibiotics as chemotherapeutic agents against *P. falciparum*. There are a number of compounds that inhibit protein synthesis by affecting ribosomal GTPase activity (Cundliffe, 1990). Application of temperature-dependent hyperchromicity with synthetic RNA substrates also provides an in vitro assay for compounds of this type. Because completion of the erythrocytic cycle only takes about 48 h for *P. falciparum*, selective effect on parasite rather than host protein synthesis is likely. Selective inhibition of protein synthesis by thiostrepton (McConkey et al., 1997) has also demonstrated that the plastid-like organelle is essential for the erythrocytic cycle of *P. falciparum*. Although the function of the organelle is as yet unknown (Wilson et al., 1996), its importance in the blood stage infection indicates a novel target for antimalarial drugs. In addition to *Plasmodium* species, the plastid-like organellar genome has been identified in other members of the phylum Apicomplexa. These include *Toxoplasma gondii* (Egea & Lang-Unnasch, 1995) and *Babesia bovis* (Gozar & Bagnara, 1995). Therefore, the plastid-encoded ribosome may be a general target for antibiotics in Apicomplexa (Beckers et al., 1995).

MATERIALS AND METHODS

Synthetic genes for the *P. falciparum* fragments were constructed by ligation of oligonucleotides in plasmid pUC2119 as described (Sampson & Uhlenbeck, 1988), with a *Fok* I site inserted at the 3′ end of the gene for run-off transcription (Rogers & Söll, 1993). Large-scale transcription with T7 RNA polymerase of *Fok* I-digested plasmid DNA was as described (Gluick & Draper, 1994), with purification by electrophoresis on denaturing polyacrylamide gels, followed by cutting the band from the gel and eluting as described (Gluick & Draper, 1994). RNA samples were renatured for 30 min at 65 °C for the A- and S-type RNAs, and for 30 min at 40 °C for the 35 and 35R rRNAs prior to thermal denaturation. Samples for binding data contained RNA in 5 mM $MgCl_2$, 100 mM NH_4Cl, 5% (v/v) DMSO ± thiostrepton (8 μM; Calbiochem). Melting data were collected on a Perkin-Elmer Lambda 4 spectrophotometer as described (Laing & Draper, 1994) with heating rates between 0.8 and 1.0 °C/min. The first derivative of absorbance at 260 nm with respect to temperature was plotted.

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