RNA (1999), *5*:939–946. Cambridge University Press. Printed in the USA.

Ribosomal RNA is the target for oxazolidinones, a novel class of translational inhibitors

N.B. MATASSOVA,[1,3] M.V. RODNINA,[1] R. ENDERMANN,[2] H.-P. KROLL,[2] U. PLEISS,[2] H. WILD,[2] and W. WINTERMEYER[1]

[1]Institute of Molecular Biology, University of Witten/Herdecke, 58448 Witten, Germany
[2]Bayer AG, Business Group Pharma, 42096 Wuppertal, Germany

ABSTRACT

Oxazolidinones are antibacterial agents that act primarily against gram-positive bacteria by inhibiting protein synthesis. The binding of oxazolidinones to 70S ribosomes from *Escherichia coli* was studied by both UV-induced cross-linking using an azido derivative of oxazolidinone and chemical footprinting using dimethyl sulphate. Oxazolidinone binding sites were found on both 30S and 50S subunits, rRNA being the only target. On 16S rRNA, an oxazolidinone footprint was found at A864 in the central domain. 23S rRNA residues involved in oxazolidinone binding were U2113, A2114, U2118, A2119, and C2153, all in domain V. This region is close to the binding site of protein L1 and of the 3′ end of tRNA in the E site. The mechanism of action of oxazolidinones in vitro was examined in a purified translation system from *E. coli* using natural mRNA. The rate of elongation reaction of translation was decreased, most probably because of an inhibition of tRNA translocation, and the length of nascent peptide chains was strongly reduced. Both binding sites and mode of action of oxazolidinones are unique among the antibiotics known to act on the ribosome.

Keywords: antibiotics; E site; ribosomal RNA; translational inhibition; translocation

INTRODUCTION

Oxazolidinones are a new class of antimicrobial agents that act against a wide spectrum of bacteria, primarily gram-positive and anaerobic ones (Slee et al., 1987; Daly et al., 1988; Brickner et al., 1996; Ford et al., 1996). Studies on the mechanism of action of oxazolidinones have shown effects on protein synthesis, whereas DNA and RNA synthesis were not affected (Eustice et al., 1988a, 1988b). Which particular step of protein synthesis is suppressed is not known. It has been shown that formation of fMet-tRNA^{fMet}, peptide bond formation, and termination are not inhibited by the antibiotics (Lin et al., 1997; Shinabarger et al., 1997). The effects on the initiation and elongation steps of protein synthesis are less clear. Cell extracts treated with an oxazolidinone (DuP 721) were severely impaired in protein synthesis on natural mRNA (Eustice et al., 1988b). In contrast, no effect was observed when synthetic mRNAs were used, such as $(GU_2)_n$ (Eustice et al., 1988b) or poly(U) (Shinabarger et al., 1997). Because translation on natural mRNA requires initiation, unlike that on synthetic mRNAs, it was suggested that initiation may be affected by oxazolidinones (Eustice et al., 1988b). On the other hand, no inhibition of fMet-tRNA^{fMet} binding to the ribosome or of fMet-puromycin formation was observed, indicating that in the presence of oxazolidinones, initiation resulted in correct positioning of the tRNA–mRNA complex in the ribosomal P site (Eustice et al., 1988a).

The precise target for oxazolidinones is not known, although binding to the 50S ribosomal subunit was reported (Lin et al., 1997). The binding of the oxazolidinone eperezolid was competitively inhibited by chloramphenicol and lincomycin, indicating overlapping binding sites. However, since the affinity of oxazolidinone was estimated to be 10 times lower than that of chloramphenicol and lincomycin, and because of the different mode of action of oxazolidinone, the effects on binding can be also explained by conformational changes of 23S rRNA, rather than by direct competition.

In the present paper, we determine the binding site(s) of oxazolidinones on 70S ribosomes using cross-linking and footprinting techniques. The results reveal defined

Reprint requests to: W. Wintermeyer, Institute of Molecular Biology, University of Witten/Herdecke, Stockumer Strasse 10, 58453 Witten, Germany; e-mail: winterme@uni-wh.de.
[3]On leave from Novosibirsk Institute of Bioorganic Chemistry, Siberian Branch of the Russian Academy of Sciences, Prospect Lavrentieva 8, 630090, Novosibirsk, Russia.

oxazolidinone binding sites on each ribosomal subunit, both involving binding to rRNA. In 16S rRNA, the oxazolidinone binding site was found in the loop closing helix 26 in the central domain. On the 50S subunit, a binding site in domain V of 23S rRNA was identified that is close to the binding sites for protein L1 and for the 3′ end of E site-bound tRNA. The inhibitory effect of oxazolidinones on the translation of natural mRNA was analyzed in a purified and highly active in vitro translation system from *Escherichia coli*. The data suggest that oxazolidinones inhibit elongation by affecting the translocation step. No effect was found on initiation or on the transition from initiation to elongation.

RESULTS

Localization of the oxazolidinone binding site by cross-linking

For cross-linking, ribosomes (0.5 μM) were incubated with an excess (2 μM) of a ^{3}H-labeled azido derivative of oxazolidinone, compound 1 (Fig. 1), and cross-linking was induced by UV light. The conditions were chosen such that the extent of cross-link was 0.1–0.15 mol reagent per mol ribosomes. The distribution of cross-linked compound between the ribosomal subunits was studied by sucrose gradient centrifugation (Stade et al., 1989), and 40 and 60% were found associated with the 30S and 50S subunits, respectively. The material sedimenting in the 30S and 50S peaks was collected, and the distribution of the radioactivity between the protein and rRNA fraction was analyzed (see Materials and Methods). Practically all cross-linked oxazolidinone was recovered in the 16S and 23S rRNA pools, whereas less then 5% of the radioactivity was found in the ribosomal protein fraction.

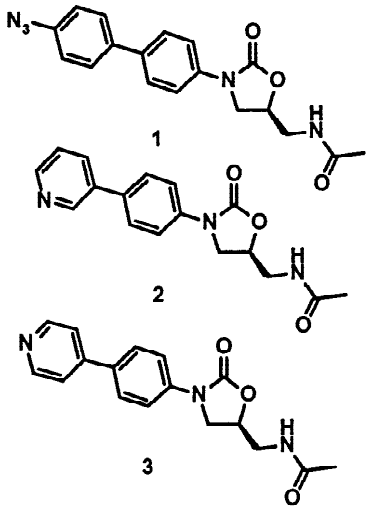

FIGURE 1. Chemical structures of three oxazolidinones used in this study. Compound 3 is identical to DuPont E3709.

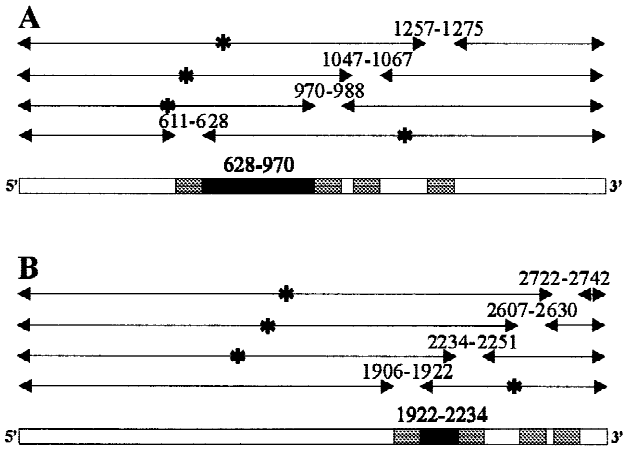

FIGURE 2. Localization of the cross-linking site of compound 1 on 16S (**A**) and 23S rRNA (**B**) by RNase H digestion. Oligodeoxyribonucleotide binding sites are shown in gray, the identified region of oxazolidinone binding in black. Of two fragments obtained after RNase H treatment (lines with arrows), the radioactively labeled fragment is indicated by an asterisk.

The sites of oxazolidinone cross-link on 16S and 23S rRNA were mapped by RNase H digestion in the presence of oligodeoxyribonucleotides complementary to selected sequences within rRNA, using rRNA isolated from ribosomes cross-linked to ^{3}H-labeled compound 1. For 16S rRNA, four oligodexyribonucleotides were used that were complementary to positions 611–628, 970–988, 1047–1067, and 1257–1275. After annealing, the regions containing RNA–DNA duplexes were digested by RNase H, yielding two rRNA fragments of varying length. The fragments were separated electrophoretically, eluted from the gel, and the radioactivity in both fragments was determined, as depicted in Figure 2A. In each case, the labeled fragment contained $>$80% of the total radioactivity (about 5,000 dpm). These data clearly show that oxazolidinone is cross-linked to the region encompassing positions 628–970 of 16S rRNA.

Analogous experiments were performed with 23S rRNA, using four oligodeoxyribonucleotides that were complementary to positions 1906–1922, 2234–2251, 2607–2630, and 2722–2742. The results of the RNase H digestion analysis show that the cross-link is localized between positions 1922–2234 of 23S rRNA (Fig. 2B).

Fine mapping of the oxazolidinone binding site on 23S rRNA was performed by primer extension using reverse transcriptase. Based on the results of RNase H digestion, oligodeoxyribonucleotide primers complementary to positions 970–988 of the 16S rRNA and 2234–2251 of the 23S rRNA were used. Because cross-linked oxazolidinone is expected to provide a steric hindrance for the reverse transcriptase, a strong stop should appear at the site of the cross-link, observable as an additional band on the sequencing gel. This was

indeed found with 23S rRNA (Fig. 3). The strongest stop was found at C2153 and weaker ones at positions U2113 and U2118.

Primer extension analysis with 16S rRNA, using a primer complementary to positions 970–988, did not reveal reverse transcriptase stops due to cross-linking in the region defined by RNase H mapping (not shown). This indicates that the cross-link to 16S rRNA did not modify positions of bases involved in base pairing, but rather other base positions or sugar residues, the modification of which would not affect the template function in reverse transcription.

Localization of the oxazolidinone binding site by footprinting

To complement the cross-linking results, we have examined the protection of bases of rRNA from chemical probing by dimethyl sulphate (DMS) in the presence of antibiotic. A large excess of antibiotic was used, and both 16S and 23S rRNAs were completely scanned by primer extension analysis to reveal all potential sites of interaction. The ribosomes were first incubated with unmodified compound 2 (Fig. 1) to form the complex, and then with DMS for modification. rRNA was isolated, and the positions of methylation were determined by primer extension using reverse transcriptase. A set of oligodeoxyribonucleotide primers was used that were complementary to regions of 16S and 23S rRNA separated by approximately 200 nt. In 16S rRNA, a strong protection of A864 was found (Fig. 4A); in the presence of compound 2, the efficiency of modification decreased by 85%. In 23S rRNA, A2114 and A2119 were similarly (80%) protected in the presence of compound 2 (Fig. 4B).

The results obtained by cross-linking and footprinting are summarized in Figure 5. On the 30S subunit, the oxazolidinone binding site is localized in the central domain of 16S rRNA, in the loop closing helix 26. On the 50S subunit, the antibiotic binds to domain V of 23S rRNA, close to the binding site of protein L1 and the ribosomal E site (Said et al., 1988; Moazed & Noller, 1989).

Mechanism of action of oxazolidinones

It has been suggested that oxazolidinones inhibit initiation and the transition from initiation to elongation (Eus-

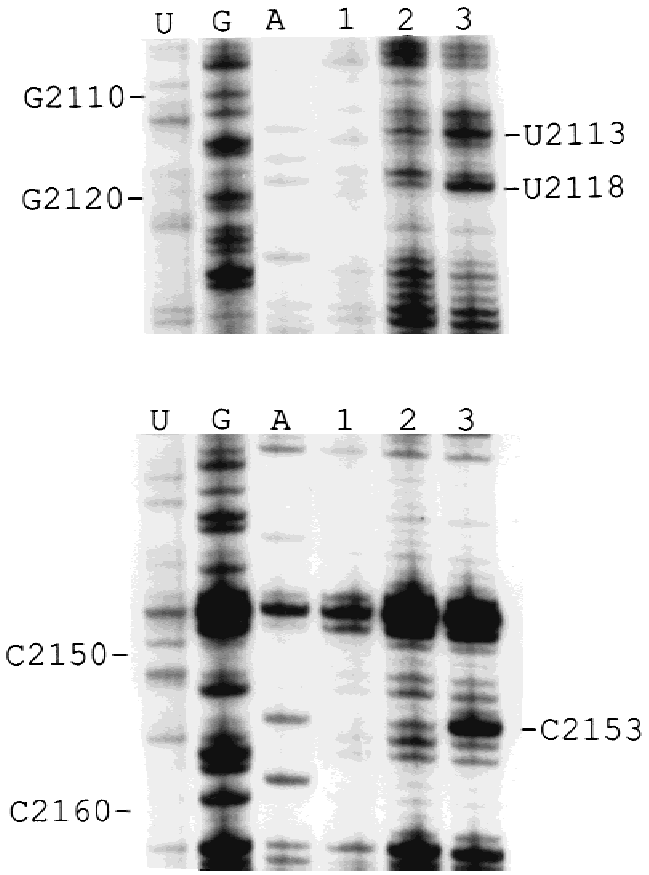

FIGURE 3. Determination of the cross-linking site of compound 1 on 23S rRNA by primer extension. Dideoxy sequencing lanes prepared from untreated 23S rRNA are marked U, G, A. Lane 1: untreated rRNA; lane 2: rRNA irradiated in the absence of compound 1; lane 3: rRNA irradiated in the presence of compound 1. The positions of oxazolidinone-mediated stops are indicated.

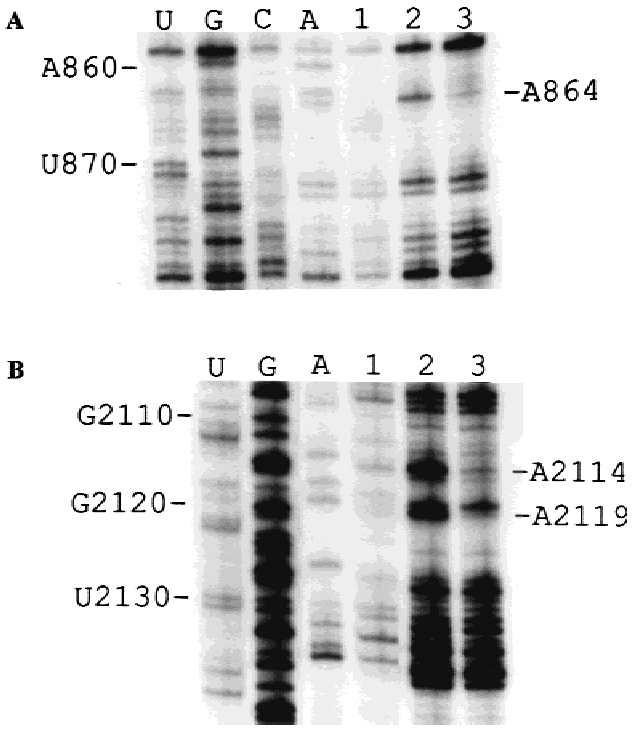

FIGURE 4. DMS footprinting of compound 2 on 16S (**A**) and 23S (**B**) rRNA. Dideoxy sequencing lanes prepared from untreated 16S and 23S rRNA are marked U, G, C, A. Lane 1: untreated rRNA; lane 2: rRNA modified by DMS in the absence of compound 2; lane 3: rRNA modified by DMS in the presence of compound 2. The positions of the stong protections are indicated.

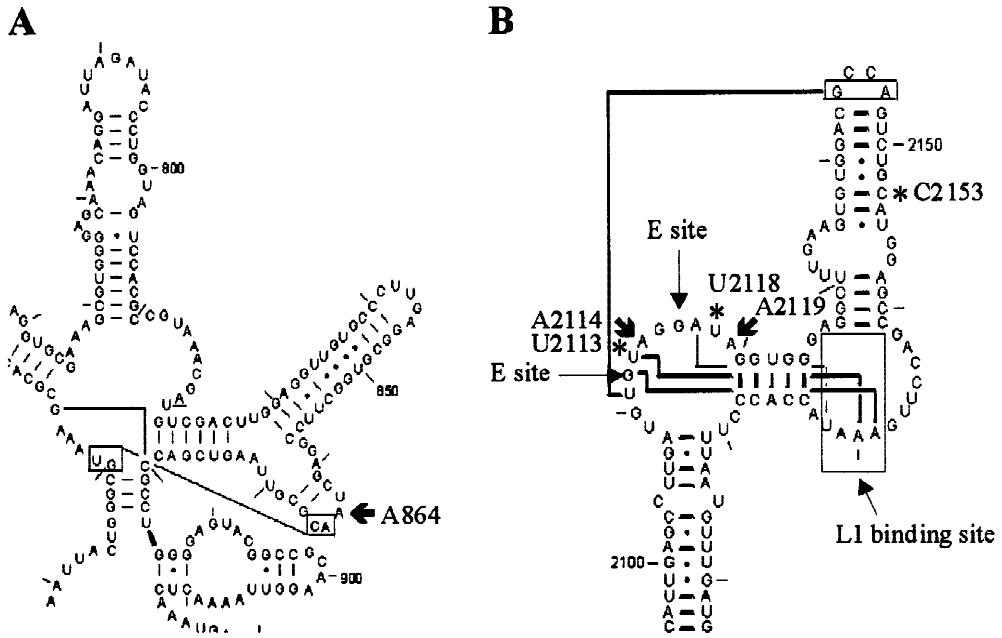

FIGURE 5. Secondary structure model of the oxazolidinone binding sites on 16S (**A**) and 23S rRNA (**B**). Cross-link (*) and footprint (↟) positions are indicated.

tice et al., 1988b). To examine these steps, we have determined the efficiencies both of f[^{3}H]Met-tRNA^{fMet} binding to the P site in the presence of mRNA and initiation factors, that is initiation complex formation, and of the binding of the ternary complex, EF-Tu•GTP•[^{14}C]Phe-tRNA^{Phe}, to the A site and the formation of the dipeptide f[^{3}H]Met[^{14}C]Phe. As shown in Table 1, there is practically no effect of oxazolidinones on these reactions, indicating that initiation and EF-Tu-dependent A-site binding of aa-tRNA are not affected. Also the time courses of the two reactions were identical in the absence and presence of antibiotics, as measured by nitrocellulose binding (initiation) or by fluorescence stopped flow (A-site binding; Rodnina et al., 1994a; data not shown).

To measure quantitatively the effect of oxazolidinones on the elongation step, we determined the average rate of translation of a natural mRNA in a translation system reconstituted from purified components. A truncated mRNA comprising the ribosome binding site and coding for the first 81 amino acids of β-lactamase was used. To avoid polysome formation, the ribosomes were programmed with an excess of mRNA. Ribosomes could synthesize one peptide chain only, because they were stalled at the last codon of the truncated mRNA, due to the lack of termination factors and a stop codon. Protein synthesis was initiated by the addition of excess EF-Tu, EF-G, and purified aa-tRNA (containing [^{14}C]Leu-tRNA) to 70S initiation complexes on β-lactamase mRNA with initiator f[^{3}H]Met-tRNA^{fMet} in the P site. The time course of translation was monitored by f[^{3}H]Met and [^{14}C]Leu incorporation into peptide. The two oxazolidinones studied, compounds 2 and 3, strongly inhibited translation (Fig. 6). Because pep-

TABLE 1. Effect of oxazolidinones on initiation, A-site binding, and transpeptidation.

Oxazolidinone	Binding (%)		fMetPhe synthesis (%)
	fMet	Phe	
None	100[a]	100[a]	100[b]
Compound 1	99	95	90
Compound 2	76	75	75
Compound 3	97	96	96

[a]9 pmol/10 pmol of ribosomes.
[b]6–8 pmol/10 pmol of ribosomes.

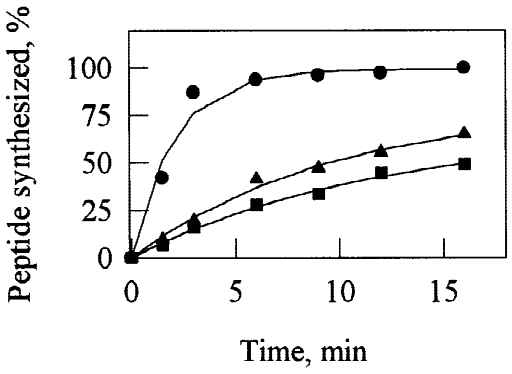

FIGURE 6. Effect of oxazolidinones on the translation of β-lactamase mRNA. (●) without antibiotic; in the presence of compounds 2 (■) or 3 (▲).

tides contained one f[^{3}H]Met and up to ten [^{14}C]Leu, the peptide chain length was determined from the ^{14}C/^{3}H ratio, and the fraction of ribosomes taking part in peptide elongation from the amount of ^{3}H in TCA-precipitable peptides. The latter number was the same in the presence and absence of oxazolidinones (Table 2), again suggesting there is no effect of the antibiotic on either initiation or the transition from initiation to elongation. In the absence of oxazolidinone, the average number of Leu per peptide was 9, which corresponds to an average chain length of 69 amino acids (of 81 possible). The rate of polypeptide synthesis was estimated by single exponential fitting to 0.64 $\pm$ 0.12 min^{-1}; therefore, the total time required for the synthesis was 1/0.64 = 1.6 min, and the time for the elongation of the nascent peptide by one amino acid is 1.6/69 = 0.023 min, yielding an average elongation rate of about 0.7 s^{-1}. In the presence of compounds 2 and 3, shorter peptides were synthesized that were about 29 and 53 amino acids long, respectively. With compound 2, the elongation rate was 0.38 $\pm$ 0.11 min^{-1} per peptide, or about 0.18 s^{-1} per amino acid (total synthesis time is 1/0.38 = 2.6 min for 29 amino acids, and 2.7/29 = 0.09 min for one amino acid). A similar value (0.17 s^{-1}) was obtained with compound 3 (elongation rate 0.20 $\pm$ 0.03 min^{-1}, total synthesis time 5.1 min, or 0.09 min per amino acid). Thus, oxazolidinones reduced the elongation rate about fourfold. Since A-site binding of aa-tRNA was not affected by oxazolidinones (see above), the reduced elongation rate is attributed to slower EF-G-dependent translocation.

DISCUSSION

The oxazolidinones bind to two distinct sites on the two subunits of the prokaryotic ribosome, in both cases to rRNA. On 23S rRNA, oxazolidinones bind to a phylogenetically highly conserved region in the vicinity of the binding site of ribosomal protein L1 (Said et al., 1988). Because of tertiary structure of the 2100–2180 region of the 23S rRNA (Fig. 5), the positions identified by cross-linking and footprinting in this region are probably close to each other. Until now, this region was not reported to be involved in antibiotic binding. This indicates that the mechanism of action of oxazolidinones is different from that of other known ribosome-targeted antibiotics.

TABLE 2. Effect of oxazolidinones on translation of β-lactamase mRNA in a purified in vitro system.

Addition	Fraction of elongating ribosomes	Average peptide length		Synthesis time (min)	Elongation rate (s^{-1})
		Leu	Amino acids[a]		
None	0.54	9	69	1.6 $\pm$ 0.3	0.72 $\pm$ 0.15
Compound 2	0.53	4	29	2.7 $\pm$ 0.8	0.18 $\pm$ 0.06
Compound 3	0.52	6	53	5.1 $\pm$ 0.8	0.17 $\pm$ 0.03

[a]Total length of peptide was 81 amino acids with the tenth Leu at position 80.

It was reported previously that oxazolidinones competed with chloramphenicol and lincomycin for binding to the 50S ribosomal subunit (Lin et al., 1997). Footprinting studies have indicated that chloramphenicol protects A2058, A2059, and A2062 (weak protections), as well as A2451 and G2505 (strong protections; Moazed & Noller, 1987), that is, in a region that is located in the P site of the peptidyltransferase center (Barta et al., 1990). Lincomycin protects the same nucleotides (A2451, G2505, and A2508) (Douthwaite et al., 1992). However, the peptidyltransferase activity of the ribosome was not affected by oxazolidinones (Lin et al., 1997). As our data show that the oxazolidinone binding site on the 50S subunit is distant from the peptidyltransferase center, the effect of the antibiotic on chloramphenicol and lincomycin interaction with the ribosome is probably indirect, indicating conformational coupling between the peptidyltransferase center and the L1 binding region of 23S rRNA, where oxazolidinones bind.

Interestingly, G2112 and G2116 of the same region of 23S rRNA were shown to be involved in the interaction of deacylated tRNA with the ribosomal E site (Moazed & Noller, 1989). Upon translocation, P site-bound deacylated tRNA moves through the E site to dissociate from the ribosome, and it has been shown that the transiently occupied E site is an important functional intermediate of translocation (Wintermeyer et al., 1990). It was shown that inhibition of the tRNA association with the E site results in strong suppression of the translocation rate (Lill et al., 1989). If the main effect of oxazolidinones on translation is due to binding to the 50S subunit, then the effect could be explained by an inhibition of tRNA release from the ribosome by interfering with the interaction in the E site.

Another interaction site of oxazolidinones was identified at position A864 of 16S rRNA. It is possible that oxazolidinone interacts with rRNA in both subunits from a single binding site. However, in the recent model of the 30S subunit (Mueller & Brimacombe, 1997a, 1997b), A864 is placed such that it would be far away from the position of protein L1. Thus, it is not excluded that there are separate binding sites for oxazolidinone on the two ribosomal subunits. A864 is close to the region of 16S rRNA where secondary binding sites for aminoglycoside antibiotics neomycin, gentamicin, and kanamycin have been reported (weak protections of G851 and C862; Moazed & Noller, 1987). Footprinting experiments using base-specific reagents or Fe(II)-EDTA have identified nearby helices 25 (positions 820–828, 872–875) and 26 (positions 858–859) as being protected by S8 (Stern et al., 1988; Powers & Noller, 1995). Although the binding site of S8 is located in helix 21

(Mougel et al., 1987; Allmang et al., 1994; Kalurachchi et al., 1997), it is possible that in the three-dimensional structure of the 30S subunit, the 860 region is also in contact with the protein (Mueller & Brimacombe, 1997b). In addition, a cross-link of the 819–859 region of 16S rRNA to IF3 has been found by chemical cross-linking (Ehresmann et al., 1986). The phylogenetic conservation of the 860 region is weak, and there are no data suggesting a direct functional role of the 860 region in translation. Thus, if oxazolidinone binding to the 30S subunit is involved in the observed inhibitory action, it may be attributed to an inhibition of long-range conformational changes of 16S rRNA that interfere with the dynamics of the rRNA junction in the vicinity of the oxazolidinone binding site. Additional effects of oxazolidinones on ribosome assembly in vivo cannot be excluded, though.

Our data show that the elongation step of translation is affected by oxazolidinones, while no influence was observed in initiation or on the transition from initiation to elongation. Furthermore, EF-Tu-dependent aa-tRNA binding to the A site and peptide bond formation were not affected. Thus, the likely functional target for oxazolidinones is the translocation step. Interestingly, not only was the average elongation rate decreased in the presence of the antibiotic, but also the overall peptide length was reduced. This indicates that the reduced translocation rate results in a premature drop-off of peptidyl-tRNA from the ribosome; the effect may be reinforced by structural elements of the mRNA that may introduce further obstacles for movement. Such effects may be difficult to observe in poly(U) translation (Eustice et al. 1988b; Shinabarger et al., 1997). Premature termination of peptide elongation may have important consequences in vivo, because incomplete and therefore functionally inactive proteins would be synthesized. As protein synthesis constitutes a major fraction of the energy-consuming activity of the bacterial cell, the accumulation of relatively small effects on the rate and processivity of elongation may have dramatic consequences for the cell. Nevertheless, the present results are also consistent with the bacteriostatic, rather than bacteriocidal, mode of action of oxazolidinone compounds.

MATERIALS AND METHODS

Materials

70S ribosomes from *E. coli* MRE 600 and EF-Tu from *E. coli* K12 were prepared as described (Rodnina & Wintermeyer, 1995). EF-G was expressed in *E. coli* JM109 using pTZ*fus* plasmid (Borowski et al., 1996). Cells were lysed in buffer A (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 mM EDTA (ethylene diamine tetraacetic acid), and 6 mM β-mercaptoethanol) in the presence of 100 μM PMSF (phenyl-methyl-sulfonyl fluoride) and 30 μM GDP (guanosine-5′-diphosphate), lysozyme (5 mg/g cells), sodium deoxycholate (12.5 mg/g cells), and DNAse I. EF-G was first purified by chromatography on Sepharose CL6B (Pharmacia) using 0–0.35 M KCl gradient in buffer A. Fractions containing EF-G were further purified by Fast Protein Liquid Chromatography (FPLC) on Superdex 75 HiLoad (Pharmacia) using buffer A containing 10% glycerol and 100 μM PMSF, and on MonoQ (Pharmacia) using a 0–0.35 M KCl gradient in buffer A with 10% glycerol. EF-G was concentrated and stored in buffer B (50 mM Tris-HCl, pH 7.5, 70 mM NH_4Cl, 30 mM KCl, 7 mM $MgCl_2$, and 1 mM dithiothreitol) with 50% glycerol.

Initiation factors were isolated from overproducing strains kindly provided by C. Gualerzi (Camerino). Cells were opened by addition of lyzozyme (5 mg/g cells), sodium deoxycholate (12.5 mg/g cells) and DNAse I in buffer C containing 20 mM Tris-HCl, pH 7.7, 60 mM NH_4Cl, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, and 0.1 mM PMSF. To wash initiation factors off the ribosomes, 1 M KCl was added to the S30 fraction, incubated 10 min, and the ribosomes were removed by centrifugation (S100). Initiation factors were purified to homogeneity by Fast-Flow FPLC on S-Sepharose using 0.2–0.8 M KCl gradient in 50 mM MOPS, pH 6.4, 0.25 mM $MgCl_2$, 0.05 mM EDTA, 5 mM β-mercaptoethanol, and 0.1 mM PMSF.

Two different mRNAs, MFTI and β-lactamase mRNA, were used for initiation and translation experiments. MFTI-mRNA contains the ribosomal binding site and a coding sequence Met-Phe-Thr-Ile…, 120 nt long in total. The plasmid pXR022 coding for mMFTI (Calogero et al., 1988) was provided by C. Gualerzi (Camerino). β-lactamase mRNA was obtained using a plasmid pBCR5-2 (G. Rauch, unpubl.); it contained the ribosome binding site followed by the coding sequence of β-lactamase. The plasmids pXR022 and pBCR5-2 were linearized by *Hin*dIII and *Sca*I, respectively. The mRNAs were prepared by run-off transcription with T7 RNA polymerase and purified by FPLC on MonoQ.

Aminoacyl-tRNA was prepared by charging total tRNA from *E. coli* (Boehringer) using 300 μM nonlabeled amino acids except leucine, 30 μM ^{14}C-leucine (560 dpm/pmol, ICN), 3 mM ATP, and *E. coli* S100 fraction in buffer B, and purified by chromatography on DE52 (Whatmann). f[^{3}H]Met-tRNA^{fMet} (3,500 dpm/pmol, ICN) was prepared and purified as described (Rodnina et al., 1994b). [^{14}C]Phe-tRNA^{Phe} from *E. coli* was purified by high-performance liquid chromatography (HPLC) on LiChrospher WP300 RP18 column (E. Merck) using a gradient of 0 to 20% ethanol in 20 mM ammonium acetate (pH 5.5), 10 mM magnesium acetate, and 0.4 M NaCl.

Compounds 2 and 3 (Fig. 1) were prepared according to Carlson et al. (1990). Compound 1 was prepared in an analogous manner. Details of the synthesis will be reported elsewhere. ^{3}H-labeled compound 1 was synthesized by coupling the appropriate amine precursor with ^{3}H-labeled acetate. The radiochemical purity of the substance was $>$98.7% as determined by HPLC, the specific activity 1,898 GBq/mmol (142 μCi/μg).

Analysis of the cross-linking site of oxazolidinone

For cross-linking, 0.5 μM ribosomes were incubated with 2 μM ^{3}H-labeled compound 1 in buffer D (20 mM HEPES, pH 7.5, 70 mM NH_4Cl, 30 mM KCl, and 7 mM $MgCl_2$) for 10 min at 37 °C, and irradiated with UV light for 90 s at 20 °C.

To minimize photodamage, a 280-nm cut-off filter (WG 280, Schott) was used. Following ethanol precipitation, ribosomal subunits were separated by 10–30% sucrose gradient centrifugation in buffer E (20 mM Tris-HCl, pH 7.5, 0.3 M NH_4Cl, 0.1 M KCl, and 1.5 mM $MgCl_2$) (Stade et al., 1989). The position of the cross-linked material in the gradient was identified by radioactivity counting.

Ribosomal subunits cross-linked to ^{3}H-labeled compound 1 were separated into ribosomal proteins and rRNA by centrifugation through 5–20% sucrose gradient in buffer containing SDS (sodium dodecyl sulfate) and EDTA (Kruse et al., 1982), and the cross-linked material identified by ^{3}H counting. For subsequent analysis, ^{3}H-labeled 16S and 23S rRNA were isolated from the cross-linked subunits according to Wollenzien et al. (1988). RNase H digestion and analysis was performed according to Bogdanov et al. (1988). The exact cross-linking position of oxazolidinone on rRNA was determined by primer extension using AMV reverse transcriptase (Promega) (Stern et al., 1988).

DMS probing

To identify the footprint position of oxazolidinone on rRNA, 0.5 μM ribosomes were incubated with 1 mM compound 2 in buffer D for 10 min at 37 °C, followed by addition of DMS and further incubation for 10 min at 37 °C. Methylated sites were determined by primer extension using AMV reverse transcriptase (Stern et al., 1988). For quantitative analysis, autoradiographic films were scanned densitometrically.

Biochemical assays

Ribosomes (0.5 μM) were incubated for 30 min at 37 °C with a 3-fold excess of mRNA in the presence of a 1.5-fold excess of IF1, IF2, IF3, and f[^{3}H]Met-tRNA^{fMet}, and 1 mM GTP (guanosine-5′-triphosphate) in buffer B, in the absence or presence of 0.2 mM oxazolidinones (compounds 2 or 3). For A-site binding experiments, MFTI-mRNA was used. Ternary complex EF-Tu•GTP•[^{14}C]Phe-tRNA^{Phe} was prepared by mixing 2 μM EF-Tu with 1 mM GTP and 1.5 μM [^{14}C]Phe-tRNA^{Phe} and 15 min incubation at 37 °C. Ternary complex was added to initiation complex, and the mixture incubated for 1 min at 37 °C. The amount of [^{14}C]Phe-tRNA^{Phe} or f[^{3}H]Met-tRNA^{fMet} bound to ribosomes was determined by nitrocellulose filtration by directly applying aliquots of the reaction mixture to the filters (Sartorius) and subsequent washing with buffer B. The filters were dissolved and radioactivity measured in QS361 scintillation cocktail. The amount of synthesized dipeptide, f[^{3}H]Met[^{14}C]Phe, was determined by HPLC on RP18 as described (Rodnina & Wintermeyer, 1995).

For translation experiments, a 70S initiation complex was formed on β-lactamase mRNA. Peptide synthesis was initiated by adding EF-Tu (5 μM), EF-Ts (3 μM), EF-G (2 μM) and purified aa-tRNA (10 μM) to initiation complexes (0.2 μM). Aliquots were withdrawn after various times of incubation at 37 °C, quenched with 0.5 M KOH, hydrolyzed for 15 min at 37 °C, and precipitated with excess 10% cold TCA. The precipitates were collected on nitrocellulose filters (Sartorius) and washed with 10 mL of cold 5% TCA. After washing with isopropanol, filters were dissolved in QS361 and counted.

ACKNOWLEDGMENTS

We thank Yuri Semenkov and Vladimir Katunin for purified tRNA, Claudio Gualerzi for *E. coli* strains overproducing initiation factors and mRNA constructs, Gabriele Rauch for the plasmid construct coding for β-lactamase mRNA, and Petra Striebeck for excellent technical assistance. The work was supported by Bayer AG, the Fonds der Chemischen Industrie, and the Alfried Krupp von Bohlen und Halbach-Stiftung.

Received February 2, 1999; returned for revision March 8, 1999; revised manuscript received April 16, 1999

REFERENCES

Allmang C, Mougel M, Westhof E, Ehresmann B, Ehresmann C. 1994. Role of conserved nucleotides in building the 16S rRNA binding site of *E. coli* ribosomal protein S8. *Nucleic Acids Res 22*:3708–3714.

Barta A, Kuechler E, Steiner G. 1990. Photoaffinity labeling of the peptidyltransferase region. In: Hill WE, Dahlberg A, Garret RA, Moore PB, Schlessinger D, Warner JR, eds. *The ribosome*. Washington, DC: American Society of Microbiology. pp 358–365.

Bogdanov A, Chichkova N, Kopylov A, Mankin A, Skripkin E. 1988. Surface topography of ribosomal RNA. *Methods Enzymol 164*: 440–456.

Borowski C, Rodnina MV, Wintermeyer W. 1996. Truncated elongation factor G lacking the G domain promotes translocation of the 3′ end but not of the anticodon domain of peptidyl-tRNA. *Proc Natl Acad Sci USA 93*:4202–4206.

Brickner SJ, Hutchinson MR, Barbachyn MR, Manninen PR, Ulaniwicz DA, Garmon SA, Grega KC, Hendges SK, Toops DS, Ford CW, Zurenko GE. 1996. Synthesis and antimicrobial activity of U-100592 and U-100766, two oxazolidinone antibacterial agents for the potential treatment of multidrug-resistant gram-positive bacterial infections. *J Med Chem 39*:673–679.

Calogero RA, Pon CL, Canonaco MA, Gualerzi CO. 1988. Selection of the mRNA translation initiation region by *Escherichia coli* ribosomes. *Proc Natl Acad Sci USA 85*:6427–6431.

Carlson RK, Gregory WA, Park C. 1990. European Patent Application 352781.

Daly JS, Eliopoulos GM, Willey S, Moellering RC. 1988. Mechanism of action and in vitro and in vivo activities of S-6123, a new oxazolidinone compound. *Antimicrob Agents Chemother 32*:1341–1346.

Douthwaite S. 1992. Interaction of the antibiotics clindamycin and lincomycin with *Escherichia coli* 23S ribosomal RNA. *Nucleic Acids Res 20*:4717–4720.

Ehresmann C, Moine H, Mougel M, Dondon J, Grunberg-Manago M, Ebel JP, Ehresmann B. 1986. Cross-linking of initiation factor IF3 to *Escherichia coli* 30S ribosomal subunit by *trans*-diamminedichloroplatinum(II): Characterization of two cross-linking sites in 16S rRNA; a possible way of functioning for IF3. *Nucleic Acids Res 14*:4803–4821.

Eustice DC, Feldman PA, Slee AM. 1988a. Mechanism of action of DuP 721, a new antibacterial agent: Effect on macromolecular synthesis. *Biochim Biophys Res Commun 150*:965–971.

Eustice DC, Feldman PA, Zajac I, Slee AM. 1988b. Mechanism of action of DuP 721: Inhibition of an early event during initiation of protein synthesis. *Antimicrob Agents Chemother 32*:1218–1222.

Ford CW, Hamel JC, Wilson DM, Moerman JK, Stapert D, Yancey RJ, Hutchinson DK, Barbachyn MR, Brickner SJ. 1996. In vivo activities of U-100592 and U-100766, novel oxazolidinone antimicrobial agents, against experimental bacterial infections. *Antimicrob Agents Chemother 40*:1508–1513.

Kalurachchi K, Uma K, Zimmermann RA, Nikonowicz EP. 1997. Structural features of the binding site for ribosomal protein S8 in *Escherichia coli* 16S rRNA defined using NMR spectroscopy. *Proc Natl Acad Sci USA 94*:2139–2144.

Kruse TA, Siboska GE, Clark BFC. 1982. Photosensitized crosslinking of tRNA to the P, A, and R sites of *Escherichia coli* ribosomes. *Biochimie 64*:279–284.

Lill R, Robertson JM, Wintermeyer W. 1989. Binding of the 3′ terminus of tRNA to 23S rRNA in the ribosomal exit site actively promotes translocation. *EMBO J 8*:3933–3938.

Lin AH, Murray RW, Vidmar TJ, Marotti KR. 1997. The oxazolidinone eperezolid binds to the 50S ribosomal subunit and competes with binding of chloramphenicol and linkomycin. *Antimicrob Agents Chemother 41*:2127–2131.

Moazed D, Noller HF. 1987. Chloramphenicol, erythromycin, carbomycin, and vernamycin B protect overlapping sites in the peptidyl transferase region of 23S ribosomal RNA. *Biochimie 69*:879–884.

Moazed D, Noller HF. 1989. Interaction of tRNA with 23S rRNA in the ribosomal A, P, and E sites. *Cell 57*:585–597.

Mougel M, Eyermann F, Westhof E, Romby P, Expert-Bezançon A, Ebel J-P, Ehresmann B, Ehresmann C. 1987. Binding of *E. coli* ribosomal protein S8 to 16S rRNA. A model for the interaction and the tertiary structure of the RNA binding site. *J Mol Biol 198*:91–107.

Mueller F, Brimacombe R. 1997a. A new model for the three-dimensional folding of *Escherichia coli* 16S ribosomal RNA. I. Fitting the RNA to a 3D electron microscopic map at 20 Å. *J Mol Biol 271*:524–544.

Mueller F, Brimacombe R. 1997b. A new model for the three-dimensional folding of *Escherichia coli* 16S ribosomal RNA. II. The RNA–protein interaction data. *J Mol Biol 271*:545–565.

Powers T, Noller HF. 1995. Hydroxyl radical footprinting of ribosomal proteins on 16S rRNA. *RNA 1*:194–209.

Rodnina MV, Fricke R, Wintermeyer W. 1994a. Transient conformational states of aminoacyl-tRNA during ribosome binding catalyzed by elongation factor Tu. *Biochemistry 33*:12267–12275.

Rodnina MV, Semenkov YuP, Wintermeyer W. 1994b. Purification of fMet-tRNA^{fMet} by Fast Protein Liquid Chromatography. *Anal Biochem 219*:380–381.

Rodnina MV, Wintermeyer W. 1995. GTP consumption of elongation factor Tu during translation of heteropolymeric mRNAs. *Proc Natl Acad Sci USA 92*:1945–1949.

Said B, Cole JR, Nomura M. 1988. Mutational analysis of the L1 binding site of 23S rRNA in *Escherichia coli. Nucleic Acids Res 16*:10529–10545.

Shinabarger DL, Marotti KR, Murray RW, Lin AH, Melchior EP, Swaney SM, Dunyak DS, Demyan WF, Buysse JM. 1997. Mechanism of action of oxazolidinones: Effect of linezolid and eperezolid on translation reactions. *Antimicrob Agents Chemother 41*:2132–2136.

Slee AM, Wuanola MA, McRipley RJ, Zajac I, Bartolomew PT, Gregory WA, Forbes M. 1987. Oxazolidinones, a new class of synthetic antibacterial agents: In vitro and in vivo activities of DuP 105 and Dup 721. *Antimicrob Agents Chemother 31*:1791–1797.

Stade K, Rinke-Appel J, Brimacombe R. 1989. Site-directed cross-linking of mRNA analogues to the *Escherichia coli* ribosome; identification of 30S ribosomal components that can be cross-linked to the mRNA at various points 5′ with respect to the decoding site. *Nucleic Acids Res 17*:9889–9908.

Stern S, Moazed D, Noller HF. 1988. Structural analysis of RNA using chemical and enzymatic probing monitored by primer extension. *Methods Enzymol 164*:481–489.

Wintermeyer W, Lill R, Robertson JM. 1990. Role of the tRNA exit site in ribosomal translocation. In: Hill WE, Dahlberg A, Garret RA, Moore PB, Schlessinger D, Warner JR, eds. *The ribosome.* Washington, DC: American Society of Microbiology. pp 348–357.

Wollenzien P. 1988. Isolation and identification of RNA cross-links. *Methods Enzymol 164*:319–329.