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Mapping of *Escherichia coli* Ribosomal Components Involved in Peptidyl Transferase Activity

(ribosomal proteins/bromamphenicol/*N*-bromoacetyl derivatives/50S subunit)

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ABSTRACT **The method of affinity labeling has been used to identify protein components of 50S ribosomal subunits involved in peptidyl transferase activity. *E. coli* 50S ribosomal subunits were mapped by reaction with the *N*-bromoacetyl analog of chloramphenicol, an antibiotic known to interact specifically with the active center of the enzyme. The synthetic analog competes with chloramphenicol in binding to 50S ribosomal subunits and inhibits peptidyl transferase activity. It attaches covalently to the ribosome under appropriate conditions and causes an irreversible loss in peptidyl transferase activity. The reagent specifically alkylates cysteine residues of proteins L2 and L27.**

The elucidation of the detailed mechanism of ribosome action in protein synthesis depends on the identification of the specific ribosomal proteins engaged in the different steps of the process. This communication describes an attempt to localize the 50S ribosomal subunit components directly involved in peptidyl transferase activity, by the method of affinity labeling. The antibiotic chloramphenicol was selected for this purpose in view of its specific interaction with the 50S ribosomal subunit at, or in the vicinity of, the active site of peptidyl transferase (1, 2). An analog of chloramphenicol suitable for affinity labeling was synthesized with an *N*-monobromoacetyl moiety replacing the naturally occurring *N*-dichloroacetyl group. The biological activity of the synthetic analog, bromamphenicol, was assessed by its ability to compete with chloramphenicol in binding to the 50S subunit, as well as by its ability to inhibit peptidyl transferase activity. Our results indicate that bromamphenicol effectively competes with chloramphenicol in binding to the 50S subunit and inhibits peptidyl transferase activity. The analog binds covalently to the 50S ribosomal subunit under appropriate conditions and causes irreversible inactivation of peptidyl transferase activity by selectively alkylating cysteine–SH groups of proteins L2 and L27 (nomenclature of ref. 3).

MATERIALS AND METHODS

Synthesis of N-Bromoacetyl Derivatives. [^{14}C]Bromamphenicol. 3.7 mg of [^{14}C]bromoacetic acid (50 Ci/mol, Radiochemical Centre) was mixed with 6.5 mg of unlabeled bromoacetic acid in 100 μl of dioxane. 12 mg of dicyclohexylcarbodiimide and 6 mg of *N*-hydroxysuccinimide were added and the mixture was left for 1 hr at room temperature. 12 mg of D-(-)-*threo*-1-*p*-nitrophenyl-2-amino-1,3-propanediol (prepared by acid hydrolysis of chloramphenicol) was added and incubation was continued for 1 hr. Dicyclohexylurea was removed by centrifugation after addition of 2 ml of ethylacetate. The clear solution was extracted twice with water and evaporated to dryness; the residue was purified by thin-layer chromatography on silica gel developed with chloroform–methanol 3:1. Unlabeled bromamphenicol was prepared according to Rebstock (4).

N-Bromo[^{14}C]Acetylphenylalanine and Its Methyl Ester. Phenylalanine and its methyl ester were coupled with the *N*-hydroxysuccinimide ester of bromo-[^{14}C]acetic acid essentially as in the preparation of [^{14}C]bromamphenicol.

Ribosome Preparation. Isolated 50S ribosomal subunits from 1 M NH_4Cl-washed ribosomes from *Escherichia coli* MRE-600 were prepared as described (5). The final preparation was dialyzed against and kept in buffer 1 containing 0.1 M NH_4Cl–1 mM $Mg(OAc)_2$–0.02 M Tris·HCl (pH 8.6). The subunits did not lose any activity when stored in liquid air for 1 month.

Assays. Activity assays were performed at the pH values indicated in the figure legends.

Peptidyl transferase activity was assayed for 9 min by a modification of the "fragment" reaction (6), with 50 μg of 50S subunits and 30,000 cpm of [^{14}C]fMet-tRNA ([^{14}C]-methionine, 222 Ci/mol, New England Nuclear Corp.). Under these conditions the rate of the reaction was directly proportional to the ribosome concentration.

Reversible binding of [^{14}C]chloramphenicol (4.91 Ci/mol, New England Nuclear Corp.) and [^{14}C]bromamphenicol (18.1 Ci/mol) was assayed for 15 min at 0° with 300 μg of 50S subunits per 100 μl of reaction mixture, essentially as described by Vogel *et al.* (7).

Covalent Binding of Various Reagents to 50S Ribosomal Subunits. 50S Subunits (25 mg/ml) were incubated in buffer 1 at 37° with ^{14}C-labeled bromamphenicol (0.3 mM, 18.1 Ci/mol), *N*-bromoacetylphenylalanine methyl ester (1.3 mM, 17.3 Ci/mol), or *N*-ethylmaleimide (0.1 mM, 10.3 Ci/mol, Schwarz/Mann). The mixtures were dialyzed overnight against 0.1 M NH_4Cl–0.01 M $Mg(OAc)_2$–0.02 M Tris·HCl (pH 7.3) to remove unbound reagent. The total uptake of reagent was determined by precipitation of the ribosomes with cold 5% Cl_3CCOOH and filtration through glass-fiber filters (Whatman GF/C). The filters were rinsed with 5% Cl_3CCOOH, then with ethanol; they were then dried. Their radioactivity was determined in a scintillation spectrophotometer with a counting efficiency of 80%.

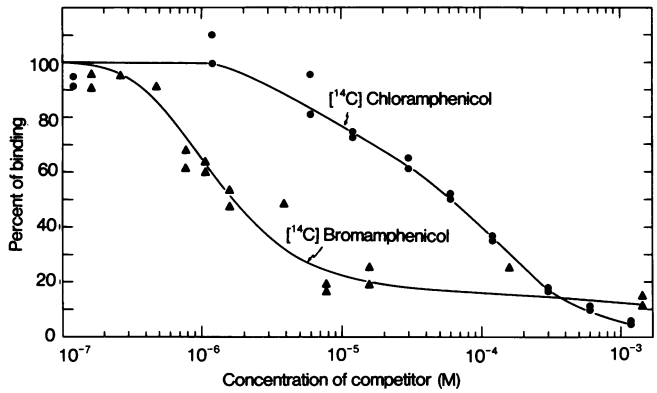

FIG. 1. Competition between chloramphenicol and bromamphenicol in binding to 50S subunits. Binding was assayed at pH 8.6. 10 μM [^{14}C]Chloramphenicol (●——●), or 10 μM [^{14}C]-bromamphenicol (▲——▲) was mixed with the indicated concentrations of unlabeled bromamphenicol or chloramphenicol, respectively. 100% Binding corresponded to 38.6 pmol of bound [^{14}C]chloramphenicol and 15.3 pmol of bound [^{14}C]bromamphenicol.

Analysis of Ribosomal Proteins. Ribosomal proteins were isolated (8) and resolved by two-dimensional acrylamide gel electrophoresis according to Kaltschmidt and Wittman (9), as modified by Avital and Elson (personal communication). 0.35–0.45 mg of the protein mixture was applied to each gel. The stained spots corresponding to individual proteins were cut out and burned in a Sample Oxidizer (Tri-Carb, model 305), and their radioactivity was determined in a scintillation spectrophotometer. The counting efficiency in this case was about 60% and the recovery of radioactivity from the gels was about 30% of the input.

RESULTS

Competition and inhibition studies

The ability of chloramphenicol and bromamphenicol to compete with each other for binding sites on 50S subunits was studied under conditions in which the covalent attachment of the synthetic analog was negligible. In these experiments the binding of [^{14}C]chloramphenicol (10 μM) was assayed in the presence of various amounts of unlabeled bromamphenicol. Similarly, the binding of [^{14}C]bromamphenicol (10 μM) was tested in the presence of unlabeled chloramphenicol. The results of such studies (Fig. 1) reveal that each of the two compounds can prevent the binding of the other, indicating that both probably share the same binding sites on the 50S subunit. However, the affinity of bromamphenicol for ribosomes appears to be 20% or less than that of chloramphenicol.

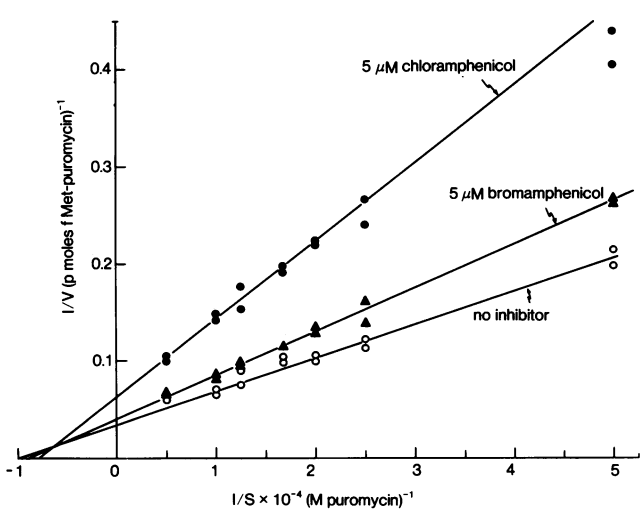

FIG. 2. Inhibition of peptidyl transferase activity by chloramphenicol and bromamphenicol. Enzymatic activity was assayed at pH 8.6. Unlabeled antibiotics were added at the indicated concentration.

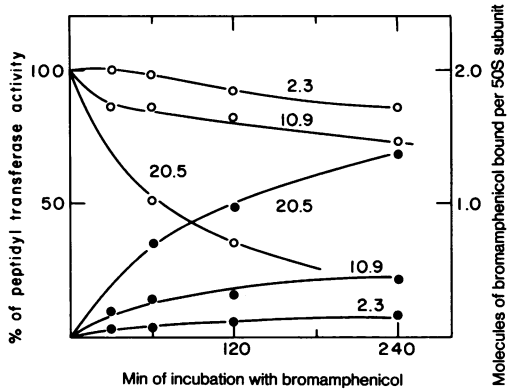

FIG. 3. Covalent binding of [^{14}C]bromamphenicol and inhibition of peptidyl transferase activity. 50S Subunits at 25 mg/ml were incubated with [^{14}C]bromamphenicol at concentrations of 36 μM, 170 μM, and 320 μM, corresponding to molar ratios of bromamphenicol to ribosomes of 2.3, 10.9, and 20.5, respectively (indicated on the curves). Samples were withdrawn at the specified times, dialyzed to remove excess reagent, and assayed for total uptake of bromamphenicol (●——●) and peptidyl transferase activity, at pH 7.3 (○——○).

The ability of the two compounds to inhibit peptidyl transferase activity was compared by measuring their effect on the rate of fMet-puromycin formation at different puromycin concentrations. The results, presented as a double-reciprocal plot (Fig. 2), indicate that bromamphenicol inhibits peptidyl transferase activity but less than chloramphenicol.

Covalent attachment of bromamphenicol to 50S subunits

Covalent attachment of bromamphenicol to 50S subunits proceeded very slowly at 0° in buffer at pH 7.3. The rate of the reaction increased considerably at 37° and when the pH was raised to 8.6. The time course of incorporation of [^{14}C]-bromamphenicol into 50S ribosomal subunits is shown in Fig. 3. Incorporation was enhanced at higher concentrations of the reagent and was accompanied by a corresponding decrease in peptidyl transferase activity (expressed relative to controls incubated in the absence of bromamphenicol). Thus, 50% of the initial enzymatic activity was lost upon the irreversible binding of 1 mol of bromamphenicol per mol of 50S subunits. Concurrent with the decrease in peptidyl transferase activity, the ribosomes also lost their ability to bind chloramphenicol or erythromycin (not shown). The ability of ribosomes to bind these antibiotics was shown (7) to be related to the ability of ribosomes to catalyze the peptidyl transfer reaction.

In order to localize the site of attachment of bromamphenicol, 50S subunits were labeled with the radioactive reagent and dissociated into their protein and RNA components.

Examination of these components showed the label to be associated mostly with the protein fraction (with less than 5% of the label in the RNA). Resolution of the protein fraction by two-dimensional acrylamide gel electrophoresis (Fig. 4) and determination of the radioactivity associated with individual proteins showed that the label was predominantly associated with two protein components, L2 and L27 (Table 1). A small amount of label in protein L26 was noted. Proteins L2 and L27 were labeled to about the same extent.

Amino-acid analysis and radioactive scanning of the acid-hydrolyzed proteins isolated from labeled 50S ribosomal subunits showed that the only radioactive component was *S*-carboxymethylcysteine, identified by its position relative to aspartic acid. Thus, bromamphenicol reacts exclusively with cysteine residues in the ribosomal proteins.

Several experiments were performed to examine the specificity of the modification by bromamphenicol. To test whether alkylation occurred at a chloramphenicol-specific site on the ribosome, the reaction with [^{14}C]bromamphenicol was conducted in the presence of chloramphenicol in 10-fold molar excess. The extent of reaction was determined after different incubation periods and was found to be the same as in the absence of chloramphenicol. Chloramphenicol, at the concentration tested, thus failed to protect ribosomes from reacting with the synthetic analog.

TABLE 1. *Distribution of label in proteins of 50S ribosomal subunits treated with various reagents*

Ribosomal protein	Bromamphenicol	*N*-Bromoacetyl-Phe methyl ester	*N*-Ethylmaleimide
		bound cpm	
L1	0	180	24
L2	480	170	965
L3	0	60	25
L4	0	40	17
L5	0	30	32
L6	40	95	81
L7 + L12	0	20	10
L8 + L9	10	10	41
L10	67	165	892
L11	50	145	286
L13	11	60	93
L14	71	85	43
L15	38	50	29
L16	11	35	26
L17	81	110	580
L18	6	55	43
L19	33	25	46
L21	2	10	0
L22	13	85	0
L23	0	75	0
L24	7	20	0
L25	0	25	0
L26	128	0	0
L27	446	50	750
L28	60	35	0
L29	0	10	0
L30	0	25	0
L32	0	10	0
L33	0	30	0

The total uptake of reagent (molecules of reagent per ribosome) corresponded to 0.5 for bromamphenicol, 0.3 for *N*-bromoacetylphenylalanine methyl ester, and 2.7 for *N*-ethylmaleimide after incubation periods of 60, 30, and 60 min, respectively.

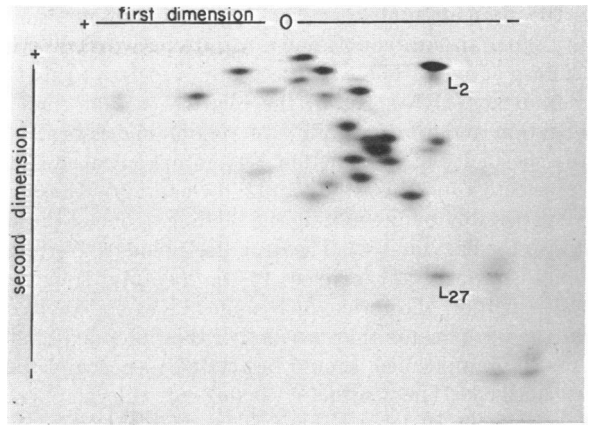

FIG. 4. Resolution of 50S subunit proteins by two-dimensional gel electrophoresis.

The specificity of the reaction was further tested by studying the effects of several nonspecific reagents: *N*-bromoacetylphenylalanine and its methyl ester and the sulfhydryl-blocking agent *N*-ethylmaleimide. None of these reagents inhibited peptidyl transferase activity. The first reagent failed to label the ribosomes, and the other two reagents bound covalently to the 50S subunits, mostly to cysteine residues. However, their labeling patterns (Table 1) were significantly broader than that of bromamphenicol, both labeling several proteins not labeled by bromamphenicol. The phenylalanine derivative failed to bind to protein L27.

To test whether bromamphenicol modification occurred at cysteine residues that also react with *N*-ethylmaleimide, the 50S subunits were treated with unlabeled *N*-ethylmaleimide and then exposed to [^{14}C]bromamphenicol. It was found that the uptake of radioactivity was considerably reduced and the subunits remained fully active. The labeling of proteins L2 and L27 was reduced by 47 and 87%, respectively. These results indicate that *N*-ethylmaleimide blocks the attachment sites of bromamphenicol, but does not in itself cause inactivation. The bound bromamphenicol must then exert a specific steric hinderance that results in inactivation of peptidyl transferase activity.

DISCUSSION

One of the most serious difficulties in identification of functionally specific ribosomal components stems from the fact that structural integrity is required to maintain ribosomes in a functional form. Omission of components or nonspecific modifications may cause inactivation of biological activity by distorting the overall structure of the ribosome, rather than directly affecting functional sites. This difficulty can be potentially overcome by use of the method of affinity labeling whereby modification should take place specifically at the functional site itself. Several recent studies have reported the use of affinity labeling to modify ribosomal components at the active center of peptidyl transferase (10–12). In all of these studies substituted Phe-tRNA was used as the affinity label. The efficiency of labeling was generally very low, rendering identification of the labeled proteins very difficult. In addition, these studies did not provide rigorous proof for the specificity of the modification.

In the present study, we have preferred to use as the affinity label an antibiotic known to interfere with peptidyl transferase activity. The advantage of molecules of this type over aminoacyl-tRNA lies in the relative ease of chemical modification and the possibility of conducting critical tests for the specificity of the reaction. Chloramphenicol, the antibiotic selected for this study, inhibits peptidyl transferase activity, possibly by preventing the binding of one of the substrates to the enzyme (13). The drug itself binds reversibly to ribosomes, probably at, or close to, the peptidyl transferase site, although an allosteric effect cannot be excluded. In any event, the components that are part of the ribosomal binding site for chloramphenicol should be related to peptidyl transferase activity. The synthetic analog of chloramphenicol used as an affinity label binds covalently to 50S ribosomal subunits, alkylating cysteine residues of protein L2 and L27 with concurrent inactivation of peptidyl transferase, and loss of the ability to bind chloramphenicol and erythromycin. Bromamphenicol attaches also to isolated 30S subunits (not shown), which do not possess a binding site for the natural analog, chloramphenicol. This observation may not be surprising in view of the ease with which sulfhydryl groups of 30S subunits react with various reagents (14, 15).

The specificity of the interaction of bromamphenicol with 50S subunits was tested in several ways. Since the alkylation reaction proceeded very slowly at 0°, it was possible to conduct inhibition and competition studies without complications arising from the irreversible binding of the reagent. Under such conditions bromamphenicol inhibited peptidyl transferase activity and competed with chloramphenicol for the same binding site(s) on the 50S subunit. Bromamphenicol in excess amounts completely abolished [^{14}C]chloramphenicol binding; however, chloramphenicol in excess amounts, although abolishing most of the [^{14}C]bromamphenicol binding, did not reduce it completely. This result suggests an additional mode of binding of bromamphenicol. It is pertinent to point out that two different modes of interaction with ribosomes have been proposed for chloramphenicol, one characterized by a high binding affinity and another by a lower affinity; both result in inhibition of peptidyl transferase activity (16). Although bromamphenicol exhibits a lower affinity than chloramphenicol to the "high-affinity" site, the synthetic analog may have a higher relative affinity for the "low-affinity" site.

The last possibility might explain the inability of chloramphenicol to interfere with the covalent binding of bromamphenicol to ribosomes. The high concentration of bromamphenicol in the alkylation experiment, 0.3 mM, compared with 10 μM in the reversible binding experiments, would be expected to enhance the binding to the "low-affinity" site. Chloramphenicol, due to its lower relative affinity for this site, would then become a poorer inhibitor of bromamphenicol binding.

The comparison of alkylation patterns indicates that bromamphenicol attacks ribosomes much more selectively than do the nonspecific reagents tested. Both *N*-ethylmaleimide and *N*-bromoacetylphenylalanine methyl ester react with more proteins than does bromamphenicol. It is of interest that *N*-bromoacetylphenylalanine methyl ester, which bears some structural resemblance to bromamphenicol, reacts only with Protein L2, not with protein L27. *N*-Ethylmaleimide reacts with both of these proteins, most probably at the same positions as does bromamphenicol but—in contrast to the antibiotic analog—does not cause inactivation of peptidyl transferase activity. This finding is in agreement with previous studies that demonstrated that free sulfhydryl groups are not essential for peptidyl transferase activity (17–19). The inactivation resulting from blockage of sulfhydryl groups with bromamphenicol or with *N*-bromoacetylpuromycin (another affinity labeling reagent, unpublished results) may point to a specific steric hinderance introduced by such reagents in a region of the 50S subunit that is critical for peptidyl transferase activity.

The selectivity of the reaction with bromamphenicol and the drastic effect of the reaction on enzymatic activity thus strongly suggest that both proteins L2 and L27 or, probably, only one of them are intimately involved in the peptidyl transferase activity of ribosomes.

NOTE ADDED IN PROOF

The use of *N*-bromoacetyl and *N*-iodoacetyl analogs of chloramphenicol for mapping of *E. coli* ribosomes has recently been reported [Bald, R., Erdman, V. A. & Pongs, O. (1972) *FEBS Lett.* **28**, 149–152]. The 50S subunit proteins that were modified were tentatively identified as L16 and L24. However, the authors have not shown that the modification affected specifically peptidyl transferase activity.

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