STIMULATION BY SULFHYDRYL-REACTIVE REAGENTS OF PEPTIDYL TRANSFERASE AND DONOR SITE BINDING ACTIVITIES IN A RETICULOCYTE SYSTEM

R. STOCKMAR, D. RINGER and H. McALLISTER
Department of Chemistry, Wayne State University, 435 Chemistry Building, Detroit, Michigan 48202, USA

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1. Introduction

It has been known for some time that exposure of ribosomes to reagents which react with sulfhydryl groups can affect their ability to carry out protein synthesis-related reactions. Specifically, we have reported that both enzymatic and non-enzymatic binding of aminoacyl-tRNA (aa-tRNA) to reticulocyte ribosomes are sensitive to mercurials and *N*-ethyl maleimide (NEM) [1, 2]. Likewise, other workers [3–5] have shown that treatment of *E. coli* ribosomes with NEM inhibits both aa-tRNA binding and polyphenylalanine synthesis.

On the other hand, a recent report by Gavrilova and Spirin [6] described the stimulation by *p*-chloromercuribenzoate (PCMB) of the "non-enzymic" (GTP- and transfer factor-independent) translocation by *E. coli* ribosomes first described by Pestka [7]. We have now extended our earlier investigation to include a study of the effects of several thiol-reactive compounds on partial reactions of protein synthesis in both prokaryotic and eukaryotic systems. Specifically, we wished to ascertain whether or not there was any involvement of sulfhydryl group(s) in two translational events which are presumed to involve only the larger ribosomal unit. These are i) the sparsomycin-induced binding of the peptidyl-tRNA analog *N*-acetyl-leucyl-oligonucleotide [8] to the ribosomal donor (D) site and ii) peptide bond formation between that tRNA fragment and puromycin (the so-called peptidyl transferase or fragment reaction as described by Monro [9]). Neither of these reactions requires GTP or transfer factors.

We wish to report here that while stimulation of these reactions was not observed in the bacterial system, reticulocyte 60 S ribosomal subunits displayed a marked increase in both activities upon exposure to several sulfhydryl-reactive compounds. The effect is similar to activation by monovalent cations at least in magnitude if not mechanism.

2. Methods

2.1. *Preparations*

Reticulocyte ribosomes were prepared essentially as described by Allen and Schweet [10] with the exceptions that the cells were lysed with 2.5 vol of 2.5 mM $MgCl_2$ and centrifugation was carried out at 100,000 *g* (avg.) for 4 hr. Subunits of these ribosomes were prepared by the method of Falvey and Staehelin [11] using the corresponding enzyme fractions from reticulocyte lysates. Bacterial ribosomes and their subunits were prepared from late log phase *E. coli* Q-13 cells (General Biochemicals, Inc.) by the method of Spirin [12]. The 50 S sununits were activated according to Miskin et al. [13]. *N*-acetyl-^{3}H-leucyl-oligonucleotide (specific activity 10.4 Ci/mmole) was prepared according to the procedure of Monro et al. [14].

2.2. *Assays*

Peptidyl transferase activity was measured using the conditions described by Monro et al. [14]. The concentration of fragment was 4.7 mM before addition of methanol. Ribosomal subunits were present in the concentration specified in each case.

The assay for sparsomycin-induced binding of *N*-acetyl-leucyl-oligonucleotide was essentially that used by Monro et al. [8]. The concentration of ribosomes is specified; that of fragment was 4.7 mM. The reaction was initiated with 0.5 vol of methanol and stopped by dilution with 3 ml of the following buffer: 33 mM Tris (pH 7.6, 21°), 267 mM KCl, 13.3 mM $MgCl_2$, 2.5 mM mercaptoethanol. The mixture was filtered immediately through nitrocellulose membranes which were then washed with 2 additional 3-ml portions of the same buffer solution. In both of these assays, radioactivity was determined by liquid scintillation techniques.

3. Results

The effects of *p*-chloromercuriphenylsulfonate (PCMS) on fragment binding and peptidyl transferase activities in both the *E. coli* and reticulocyte system are shown in fig. 1. In the case of the bacterial ribosomal subunits, neither activity is greatly affected at reagent conc. up to 1 mM. It has previously been reported [9] (and these data confirm) that peptidyl transferase activity in the bacterial system is relatively insensitive to low concentrations of mercurials. It should be mentioned that the small deviation from linearity seen at PCMS conc. of less than 0.1 mM is entirely reproducible. Likewise, binding of the oligonucleotide fragment to 50 S subunits is not severely inhibited, although almost 20% of the activity is lost at 0.1 mM PCMS. We have also found that higher levels of PCMS can strongly inhibit both of these reactions (data not presented).

The behavior of reticulocyte ribosomal subunits presents another picture entirely. In this case (fig. 1) the effect of PCMS is complex, with lower levels stimulating and higher levels inhibiting both activities in a nearly parallel manner. We assume that these compound curves reflect separate reaction events, with stimulation eventually being overwhelmed by the inhibitory effect.

In order to test whether or not the observed enhancement did indeed reflect involvement of sulfhydryl groups, we pretreated the 60 S subunits with other reagents which are more or less specific in their reaction with thiols. These results are presented in table 1. It can be seen that NEM and the alkyl and aryl disulfides hydroxyethyl disulfide and 5,5′-dithiobis(2-nitrobenzoate) all profoundly affect the reactions in question.

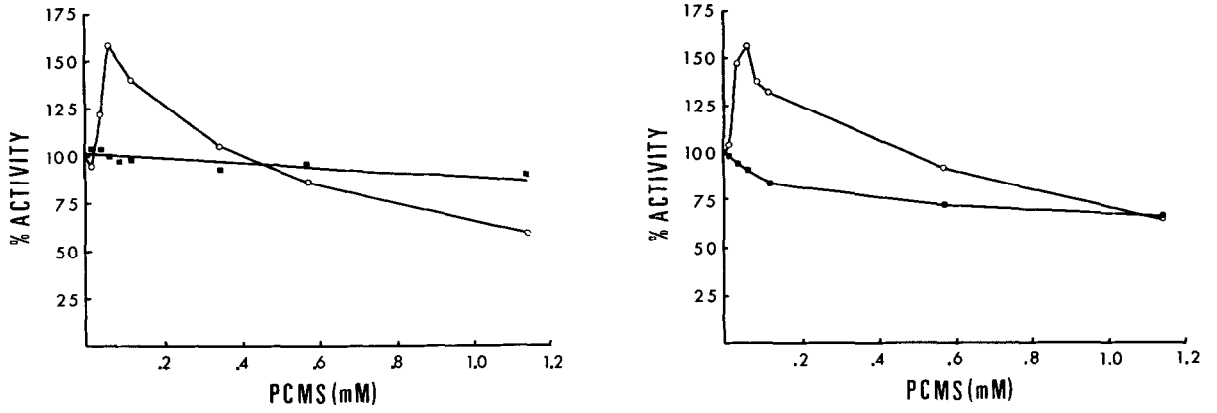

Fig. 1. Effects of PCMS on a) peptidyl transferase and b) sparsomycin-induced fragment binding. Preincubation was carried out for 30 min at 0° in 267 mM KCl, 33 mM Tris (pH 7.6 at 21°), 13.3 mM $MgCl_2$ in the presence of various concentrations of PCMS. Activity is expressed as % of control samples which were preincubated without the reagent. Control values in the peptidyl transferase assay were 55 and 67 femtomoles (fmoles) of fragment reacted in 15 min at 0° per A_{260} unit of reticulocyte and *E. coli* ribosomal subunit, respectively. The corresponding values for fragment binding were 67 and 240 fmoles of fragment bound in 15 min at 0° per A_{260} unit of subunits. All values were adjusted for radioactivity present when the appropriate antibiotic was omitted (2–8 fmoles per A_{260} unit). The conc. of subunits before addition of methanol was 12 and 15 A_{260} units per ml for peptidyl transferase and 4.8 and 6.0 A_{260} units per ml for binding reactions using 60 S and 50 S subunits, respectively. (o–o–o): 60 S; (■–■–■): 50 S.

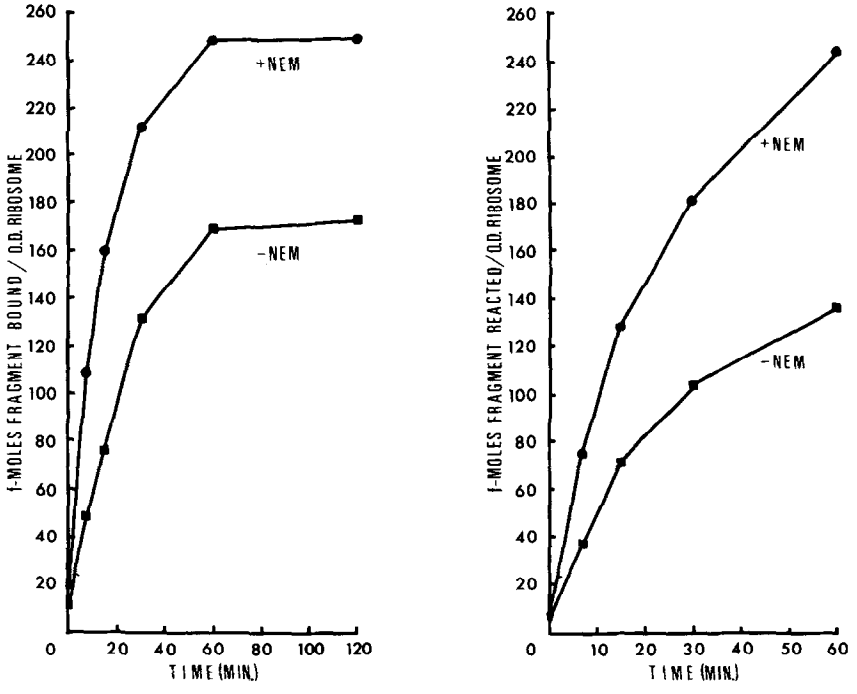

Fig. 2. Effect of preincubation of 60 S ribosomal subunits with NEM on the time course of a) sparsomycin-induced binding of fragment, and b) peptidyl transferase activity. Preincubation conditions were as described in table 1.

Further, as can be seen in fig. 2, the stimulation by NEM was apparent at all time intervals tested. For those experiments, ribosomes were preincubated with NEM; mercaptoethanol was then added to consume unreacted maleimide and then the remaining components of the reaction mixtures were added.

Omission of mercaptoethanol after NEM treatment or addition of only mercaptoethanol to the ribosomes did not alter the observed results.

Several groups have reported increases in peptidyl transferase activity by 37° incubation of certain ribosome preparations in buffers containing relatively high concentrations of monovalent cations. For instance, bacterial 50 S subunits whose peptidyl transferase activity had been destroyed by prolonged exposure to solutions of low ionic strength could be reactivated by such high salt treatment [13]. Also, monomeric ribosomes from mammalian muscle tissue have been shown to display increased peptidyl transferase activity upon incubation with 880 mM KCl [15]. Even though we had been unsuccessful in restoring peptidyl transferase activity to inactivated reticulocyte 60 S subunits, we were curious to know whether buffers of high ionic strength could replace NEM in the activation phenomenon. As can be seen in table 2, KCl at conc. of 100 mM or higher was capable of stimulating the fragment reaction, but only if the treatment included incubation at 37°. Also, the conc. of KCl apparently has no effect on the 0° activation by NEM, at least under these conditions. Sparsomycin-induced binding of *N*-acetyl-leucyl-oligonucleotide was also observed to respond in an almost identical manner (data not shown) which again demonstrated the fundamental similarity of these 2 activities. Further, other control experiments revealed that neither PCMS nor NEM could restore peptidyl transferase or fragment binding activity to bacterial 50 S subunits. In support of the findings of Vogel et al. [16] and in contrast to those of Cerna [17], we were unable to detect any fragment binding to inactivated bacterial 50 S subunits.

Table 1
Reticulocyte 60 S ribosomal subunits display enhanced peptidyl transferase and fragment binding activities if pretreated with sulfhydryl-reactive reagents.

Conditions of preincubation	Peptidyl transferase	Fragment binding
Control (ribosomes alone)	64	80
Control + NEM (5.6 mM)	130	163
Control + DTNB (1.0 mM)	125	176
Control + HED (3.0 mM)	119	154

Preincubation was carried out for 30 min at 0° in 267 mM KCl, 33 mM Tris (pH 7.6, 21°), 13.3 mM $MgCl_2$ in the presence of NEM, 5,5′-dithiobis(2-nitrobenzoate)(DTNB), hydroxyethyl disulfide (HED), or no added reagent. Activity in each case is expressed as fmoles of *N*-acetyl-leucyl-oligonucleotide reacted (or bound) per A_{260} of 60 S subunits in 15 min at 0°. Each value in the table reflects an adjustment for the radioactivity present when the appropriate antibiotic was omitted (2–8 fmoles per A_{260} unit). The conc. of subunits before addition of methanol was 14 and 5.6 A_{260} unit per ml in the peptidyl transferase and binding reactions, respectively.

Table 2
Activation of peptidyl transferase by KCl and failure of KCl to affect NEM-induced activation.

Conditions of preincubation	[KCl] during preincubation (mM)			
	20	100	287	820
Ribosomes 0°	49	56	54	54
Ribosomes + NEM 0°	95	99	95	95
Ribosomes 37°	47	108	99	104

Preincubation of 60 S subunits was carried out for 30 min in 50 mM Tris (pH 7.6, 21°), 13.3 mM $MgCl_2$, with KCl and temperature as indicated. Activity is expressed as in table 1. The concentration of subunits before methanol addition was 12 A_{260} units per ml.

4. Discussion

Enhancement of enzyme activities by treatment with mercurials has been known for some time (e.g. [18]), and in a few instances mechanisms have been postulated [19]. The recent report by Gavrilova and Spirin concerning PCMB-stimulated polyphenylalanine synthesis marks the first well-documented account of such an effect of a cell-free system of protein synthesis. For several reasons, we are unable to definitively state whether or not the stimulation by NEM and PCMS of peptidyl transferase and binding activities which we report here is related to that observation. Beyond the fact that the assay conditions differ in many ways, those authors were using monomeric bacterial ribosomes, while the stimulating effects which we observed were confined to eukaryotic ribosomal subunits. Also, they were measuring the synthesis of oligo- and polypeptides so a cyclic process involving repeated translocation on the same ribosome must have been involved. To date, however, no one has demonstrated that the fragment reaction occurs more than once per ribosomal subunit present (although Monro has reported oligopeptide synthesis by 50 S subunits using intact aa-tRNA molecules [20]). Thus while there may indeed be a common basis for both sets of findings, other experiments will be required to ascertain their relationship.

From the data presented in table 2, it is tempting to speculate that treatment of 60 S subunits with NEM is accomplishing at 0° the same thing that exposure to higher ionic strengths does at 37°. This might occur by inactivating (or making inaccessible) some group(s) which under the rather unusual conditions of the peptidyl transferase and fragment binding reaction (33% methanol) would permit binding of the substrate to the subunit.

At the present time, it is not possible to establish whether or not there exists in the reticulocyte ribosomal subunit a single sulfhydryl group in a stearically unfavorable position or if instead the NEM activation reflects an overall conformational change induced by a general reduction in the content of free thiols.

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