EFFECT OF A BIFUNCTIONAL IMIDOESTER ON DISSOCIATION OF 70S RIBOSOMES IN ESCHERICHIA COLI

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SUMMARY

Treatment of the 70S ribosome from Escherichia coli with diethyl malonimidate dihydrochloride, a bifunctional imidoester, was found to result in the formation of crosslinkage between the two subunits. The 70S complex thus obtained no longer dissociates into 50S and 30S particles at 0.5mM Mg^{2+} concentration, but do so at lower concentrations (0.1mM), suggesting the release of protein(s) involved in the inter-particle cross-linkage from one or both ribosomal subunits.

INTRODUCTION

Bacterial ribosomes have a complex structure, consisting of about 55 different proteins and three distinct species of RNA for 70S particles. Because of its complexity, as yet little information is available on the three dimensional organization of ribosomes. Physical methods such as electron microscopy and X-ray diffraction have so far revealed little information about the internal structure.

The use of bifunctional imidoesters in defining topography of the ribosome has recently been reported from several groups (1,2). Bickle et al. (2) have analyzed the spatial arrangement of a few proteins of 30S subunit by using bis- (methyl) suberimidate. This approach, combined with studies on reconstruction of the ribosomal subunits (3), seems to be effective in further analysis of internal structure of the ribosome.

By using diethyl malonimidate (DEM) that gives little crosslinkage between 30S proteins, we have obtained the cross-linked 70S complex that cannot dissociate into 50S and 30S subunits even at low Mg^{2+} concentrations (4).

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Such complexes were formed only when treated by certain species of imidoesters. The complex formation was strongly promoted by NH_4^+ ion. This effect of NH_4^+ ion seems to be explained in part by a conformational change of the ribosome that might increase reactivity of amino groups at the contact region toward imidoesters, or bring two NH_2-groups on the ribosome to a distance favorable for malonimidation. These complexes were presumably formed by a covalent crosslinkage between 50S and 30S subunits, because of its stability at low Mg^{2+} concentration. In support of this presumption, it will be shown in this paper that the complex formed by amidination is consisted of equimolar amounts of the two ribosomal subunits. Furthermore, the complex was found to dissociate completely into 50S and 30S particles at still lower Mg^{2+} concentration such as 0.1 mM.

MATERIALS AND METHODS

Strains, media and growth conditions. E. coli K12 strains Q13 and W3350 were used. The former was grown in a medium containing (in g/l) : 5 NaCl; 2 glucose; 20 polypeptone; and neutralized with NaOH. All cultures were grown at 37°C with vigorous aeration, harvested at the middle-to-late log phase, and frozen until use. The latter strain was grown in medium M9 supplemented with glucose (0.4%), $MgSO_4$ (1 mM), $CaCl_2$ (0.1 mM) and ^{14}C-amino acids (chlorella hydrolyzate, 0.1 mC/l) under otherwise similar conditions.

Ribosome purification. Ribosomes were prepared by the procedure described elsewhere (5) that includes DNase (1 μg/ml) digestion, sodium deoxycholate (0.2%) treatment and washing with high-salt buffer (0.5M ammonium acetate, 0.05M KCl, 0.1M Tris- HCl, pH 7.6, 0.01M magnesium acetate, 6 mM β-mercapto-ethanol).

Preparation of Imidoesters. Ethyl acetimidate HCl (EA) and diethyl malon-imidate 2HCl (DEM) were prepared by the method of Pinner (6) as modified by McElvain and Schroeder (7). Purity of the reagents was confirmed by element analysis and melting point measurement.

Reaction conditions. Amidination of ribosomes was performed either in mixture

A (11 mM magnesium acetate, 73 mM ammonium acetate, 60 mM Tris-HCl, pH 7.6, 6 mM β-mercaptoethanol, 73 A_{260nm} units/ml ribosomes and 240 mM DEM) or mixture B (19 mM magnesium acetate, 85 mM ammonium acetate, 9 mM Tris-HCl, pH 7.6, 6 mM β-mercaptoethanol, 27 A_{260nm} units/ml ribosomes and 240 mM DEM). Reaction was performed at room temperature ($15^{\circ}C \sim 20^{\circ}C$) for 15 or 20 min. The pH was kept between 8.0 and 8.2 by adding 5M KOH or 1N HCl. The reaction mixture was then dialyzed against dissociation buffer (10 mM Tris-HCl, pH 7.6, 6 mM β-mercaptoethanol) containing various concentration of Mg^{2+} ion. Dialysate was fractionated by sucrose gradient centrifugation in dissociation buffer with 0.5 mM Mg^{2+}.

RESULTS AND DISCUSSION

Treatment of native 70S ribosomes with DEM was found to result in the formation of the 70S complex stable at 0.5 mM Mg^{2+}. As shown in Fig. 1 (a), the complex accounts for 46% of total absorption, as judged by the sucrose gradient patterns of the treated ribosomes after 15 hours dialysis against buffer containing 0.5 mM Mg^{2+}. If dialysis was performed under lower Mg^{2+} (0.1 mM), however, no ribosomes sedimenting in the 70S region were found, suggesting that the 70S complex dissociated completely into particles of 50S and 30S. Dissociated 50S particles thus obtained were unstable, and were found to be converted to 42S particles upon prolonged storage. The DEM-treated ribosomes dialyzed at 0.5 mM Mg^{2+}, were rather stable during storage at $4^{\circ}C$. Although the 70S complex gradually dissociated into 50S and 30S particles with increasing time of storage, 28% of total absorption still remained at the 70S region even after 10 days (Fig. 1 (b)).

Thus, stability of the 70S complex is strongly dependent upon Mg^{2+} concentration. At 0.5 mM Mg^{2+}, it is stable in spite of the fact that either native 70S ribosomes or EA (monofunctional reagent) -treated ribosomes dissociate completely to the component subunits. Therefore, the 70S complex probably represents a complex of 50S and 30S particles covalently bridged by bifunctional imidoesters. Since covalent linkages should not break at low Mg^{2+} concentration, the observed dissociation of 70S complex at 0.1 mM Mg^{2+} might be attributed to

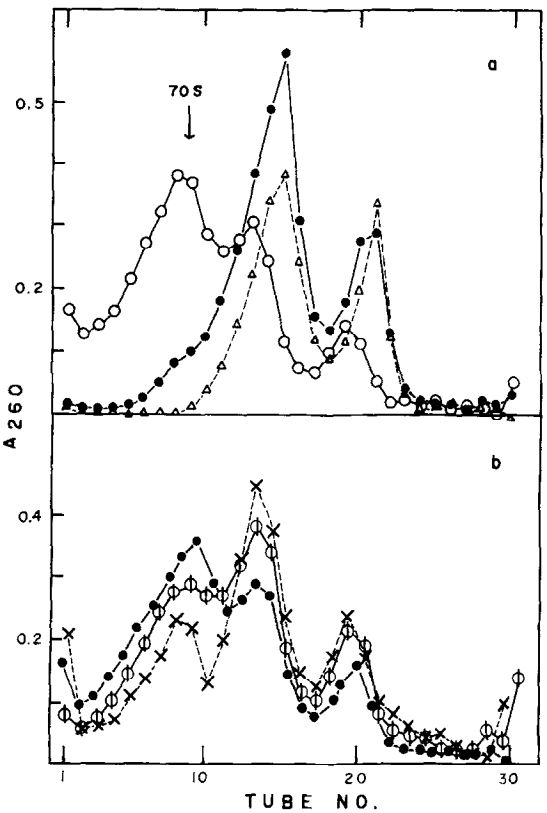

Figure 1 Sucrose density gradient patterns of amidinated ribosomes. Imidoesters (DEM) were neutralized with KOH and were added immediately to the ribosome preparation. Reaction was performed in mixture A for 15 min. and the reaction mixture was dialyzed against dissociation buffer at 4°C. Dialysate was layered on top of a 4.8 ml 5~20 % linear sucrose gradient in dissociation buffer with 0.5 mM Mg^{2+}. The gradient was spun for 130 min. at 40,000 rev./min. (a) Reaction mixture was dialyzed for 15 hr against buffer with 0.5 mM Mg^{2+} (○—○) or 0.1 mM Mg^{2+} (●—●). Untreated ribosomes were similarly dialyzed at 0.5 mM Mg^{2+} (△--△). (b) DEM-treated ribosomes that had been dialyzed against buffer with 0.5 mM Mg^{2+} were stored at 4°C, and sucrose gradient sedimentation was performed after 1.9 days (●—●), 6.4 days (⊕—⊕) or 10.7 days (X--X).

one of the following: (a) Some of the 50S proteins cross-linked to the 30S particles have been removed from the 50S particles; (b) Some of the 30S proteins cross-linked to the 50S particles have been removed from the 30S particles; (c) Covalently linked proteins connecting 50S and 30S particles came off the ribosomes.

Though these possibilities are not mutually exclusive, we first examined the possibility (a), as below.

The 70S ribosomes consisting of 50S subunits labeled with ^{14}C-amino acids

and unlabeled 30S subunits were treated with DEM under the same conditions used for the native ribosomes. The reaction mixtures were dialyzed against buffer with 0.5 mM and 0.1 mM Mg^{2+}, and were analyzed by sucrose gradient centrifugation (Fig. 2). The results obtained are essentially identical with those found with native ribosomes. Reduction of Mg^{2+} concentration to 0.1 mM again resulted in dissociation of the 70S complex into two particles. It should be noted, however, that disappearance of radioactivity at the 70S region was accompanied by the appearance of small but significant amounts of radioactivity at the 30S region and at the top of the gradient (Fig. 2 (c)). Similar results were obtained in two independent experiments. Ribosomes treated in the same way but without DEM did not result in any "shift" of radioactivity (Fig. 2 (d)). Thus the observed radioactivity at the 30S region and at the top of the gradient might be related to the mechanisms (a) and/or (c) stated above. It is quite unlikely that any shift is due to non-specific degradation of the reformed ribosomes or derived 50S particles. Only a slight shift of radioactivity to the 30S region can be seen when dialysed at 0.5 mM Mg^{2+}. Gradual dissociation of the cross-linked 70S complex occurring during storage at 0.5 mM Mg^{2+} (see Fig 1 (b)) might be responsible for the latter observation.

From the sedimentation pattern of Fig 2 (d), the ratio of radioactivity to absorbance at the 50S peak has been calculated (Table 1). Using this value, the relative absorption of 50S to 30S subunits within the cross-linked 70S complex was estimated to be about 2, the expected value for the equimolar complex (Fig. 3).

The results presented above strongly suggest that bifunctional imidoester (DEM) can form a covalent linkage between 30S and 50S ribosomal particles of *E. coli*, and that the cross-linked 70S complex dissociates completely to the component particles at extremely low Mg^{2+} concentration, such as 0.1 mM. Under these conditions, some of the proteins participating in the linkage seem to be detached from one or both of the ribosomal particles. The present evidence suggests that about 5% of ^{14}C-radioactivity (50S proteins) associated with the cross-linked 70S complexes can be shifted to 30S region. If specific 50S proteins are assumed

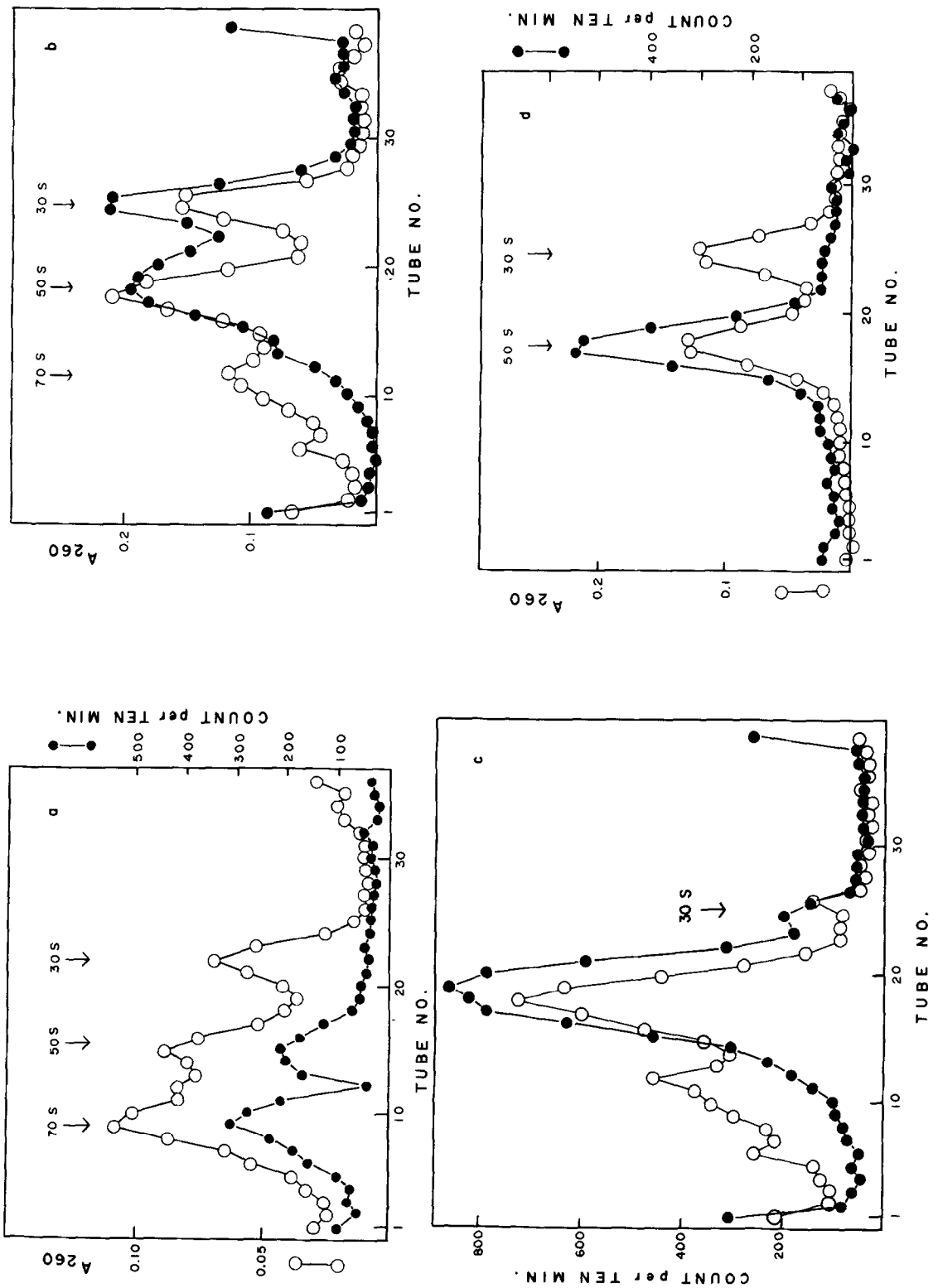
a
b
c
d
70 S
50 S
30 S
TUBE NO.
A260
COUNT per TEN MIN.

Table 1
Ratio of radioactivity to absorption of each fraction in the 50S region

TUBE No.	COUNT per TEN MIN.	A_{260}	$\frac{\text{COUNT}}{A_{260}}$
15	168	0.035	4,800
16	355	0.074	4,800
17	545	0.118	4,600
18	531	0.120	4,400
19	397	0.079	5,000
20	227	0.039	5,800
Average value			4,900

The data were taken from the experiment shown in Fig. 2 (d).

to come off with the 30S particles, this would mean the removal of one or two proteins from the 50S particles. As proteins participating in the linkage are likely to be located in the region of contact between two particles, further study on the mechanism of this dissociation may lead us to a successful separation and identification of these proteins.

Figure 2 Malonimidation of ribosomes consisting of ^{14}C-labeled 50S and unlabeled 30S particles. (a) ^{14}C-labeled 50S particles were purified from cells of W3350 grown in the presence of ^{14}C-amino acid mixture and were freed of 30S particles by repeated sucrose gradient centrifugations. They were mixed with a slight excess of unlabeled 30S particles from strain Q13 in the standard buffer (20 mM magnesium acetate, 90 mM ammonium acetate, 10 mM Tris-HCl, pH 7.6, 6 mM β-mercaptoethanol), and the mixture was incubated at 37°C for 20min. Both absorption at 260 nm (○—○) and radioactivity (●—●) were followed after sucrose-gradient centrifugation to check the formation of associated ribosomes. (b) and (c) Ribosomes were malonimidated for 20 min. in the reaction mixture B, and were dialyzed against buffer with 0.5 mM Mg^{2+} (○—○) or 0.1 mM Mg^{2+} (●—●) for 9 hr. Details of the procedure are given in Fig. 1 and in Materials and Methods. Absorption patterns are given in (b) and radioactivity in (c). (d) Untreated ribosomes were dialyzed for 9 hours against buffer with 0.5 mM Mg^{2+} and were examined by sucrose-gradient centrifugation for absorption (○—○) and radioactivity (●—●). Radioactivity of each fraction was counted for two hours in these experiments.

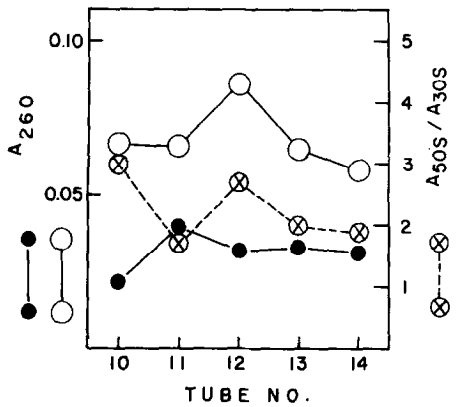

Figure 3 Relative absorption of each subunit contained by the cross-linked 70S complex. Absorption of 50S particles (○—○) within the cross-linked 70S complex shown in Fig.2b was calculated using the ratio of radioactivity to absorption obtained from Fig. 2 (d). Absorption of 30S particles (●—●) was given by subtracting the 50S value from the total absorption. The ratio of absorption (50S to 30S) is represented by ⊗--⊗ .

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