FORMATION OF ACTIVE HYBRIDS FROM SUBUNITS OF MUSCLE RIBOSOMES FROM NORMAL AND DIABETIC RATS*

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The formation of hybrids from the ribosomal subunits of bacteria has aided the localization of defective ribosomal functions such as the streptomycin-sensitive site.[1, 2] Hybridization should be useful too in the analysis of the function of ribosomes from higher organisms; however, an efficient method for the re-formation of active ribosomes from subunits has not hitherto been available. We have now found a means of preparing mammalian ribosomal subunits that allows their efficient reassociation to form active 80*S* particles and have applied the hybridization technique to a study of ribosomes from normal and diabetic animals.

Preparations of ribosomes from the muscle of alloxan-diabetic rats contain a smaller number of polysomes and are less efficient in the synthesis of protein than ribosomes from normal animals.[3–7] The injection of insulin into diabetic rats causes the reaggregation of the ribosomes and restores their activity; the action of the hormone does not require synthesis of RNA.[5] These observations have led to the view that insulin increases the ability of ribosomes to bind and translate messenger RNA (mRNA), possibly by a modification of ribosomal structure. We have now attempted to determine which of the two ribosomal subunits carries the hormone-sensitive site.

Materials and Methods.—The following have been described in detail before: the source of the materials,[5, 6] the care and alloxan treatment of the rats (weight range: 140–180 gm),[5, 6] the determination of the sedimentation coefficients of ribosomal particles on linear sucrose gradients,[7] the preparation of transfer RNA (tRNA) acylated with one radioactive and 19 nonradioactive amino acids,[3] and the method of assaying protein synthesis by ribosomes.[3, 6] Ribosomes were prepared from skeletal muscle by a modification of the method of Florini and Breuer[8] that avoided the use of high KCl concentrations.

Preparation of ribosomal subunits: Ribosomes were suspended at 0° in medium A (80 mM KCl, 12.5 mM $MgCl_2$, 50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.8) containing 20 mM β-mercaptoethanol. The concentration of potassium was adjusted to 1 *M* by addition of a stock solution containing 2.5 *M* KCl and 10 mM $MgCl_2$. The resulting suspension was warmed at 37° for 3–5 min, a sample was retained (the KCl-treated control), and 2 ml of the remaining ribosomal suspension (containing 40–60 OD_{260} units) were layered on 28 ml of a 10–30% linear sucrose gradient in medium B (20 mM β-mercaptoethanol, 850 mM KCl, 15 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.8) at room temperature. After centrifugation in a Spinco SW 25.1 rotor at 22,500 rpm for 5 hr at 27–29°, the gradients were fractionated with a model D density gradient fractionator (Instrument Specialties Co., Inc.) and analyzed at 254 mμ with a model UA-2 UV analyzer, and the subunit fractions were collected. The KCl-treated controls were diluted 1 in 10 with sucrose solution (20%, containing medium B) and allowed to stand at room temperature throughout the centrifugation and fractionation of the gradients.

Reassociation and hybridization of ribosomal subunits: Subunit fractions were either combined directly after fractionation without adjustment of the concentration of ions and then assayed (direct assay), or they were combined and dialyzed overnight at 4° against medium A containing 6% sucrose and 10 mM β-mercaptoethanol before having their activity tested. Reactivation occurred when the direct method was used, so reassociation must be rapid and specific; however, the results were somewhat variable and suf-

fered the additional limitation that the extent of reassociation could not be analyzed easily on sucrose gradients. For these reasons, the latter method (dialysis of ribosomal subunit fractions and mixtures) was generally used. Further, it was found that results were more consistent if the subunit fractions were combined before dialysis.

The 60*S* and 40*S* subunit fractions were combined in the proportion of 2.5:1 (OD_{260}). Dialyzed samples were analyzed on 15–30% linear sucrose gradients in medium A by centrifugation at 27–28°. The proportions of the individual ribosomal components were determined by planimetric analysis of the OD_{254} tracings.

Results.—Dissociation of muscle ribosomes by high concentrations of KCl: Ribosomes from the muscle of normal and diabetic rats formed particles of approximately 40*S* and 60*S* when exposed to 0.8–1.0 *M* KCl at 25–30° (Fig. 1). These 40*S* and 60*S* subunits contain predominantly 18*S* and 28*S* RNA, respectively (Martin, Low, and Wool, unpublished results). The 60*S* and 40*S* subunits were formed in the ratio of 2.5:1 (OD_{254}). Ribosomes from diabetic rats were more susceptible to dissociation than those from normals; the latter retained particles that sedimented with a coefficient greater than 60, most notably a component of approximately 75*S*.

When the purity of the subunits was determined by reanalysis on sucrose gradients in medium B (850 mM KCl), they appeared free of contamination (results not illustrated); however, when analyzed on sucrose gradients in medium A (80 mM KCl), the 60*S* fraction contained some 80*S* monomers (Fig. 2). The origin of the 80*S* monomers contaminating the 60*S* fraction is not certain; they could have formed either from dimers of the 40*S* subunit that cosedimented with the 60*S* particle or from expanded ribosome monomers that sedimented with a coefficient of 60 at higher concentrations of potassium.

Reassociation of ribosomal subunits: When the 40*S* and 60*S* ribosomal subunits were combined and dialyzed against medium A to remove the excess KCl, they reassociated to form 80*S* particles (Fig. 2).

Activity of the reassociated particles: The subunit fractions and the reassociated particles were assayed for their ability to catalyze protein synthesis. In the absence of added template RNA, very low activity was observed; we therefore repeated the assay using polyuridylic acid to direct the synthesis of polyphenylalanine.

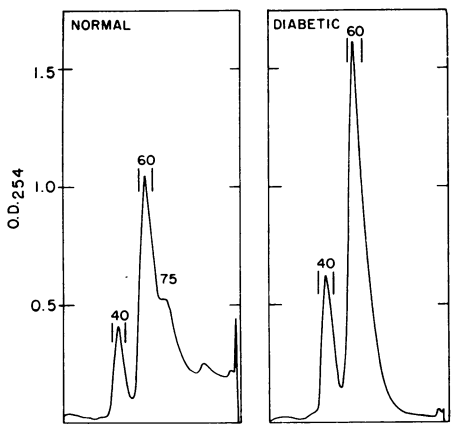

FIG. 1.—Preparation of subunits from muscle ribosomes of normal and diabetic rats. Ribosomes dissociated in 1 *M* KCl were layered on 10–30% linear sucrose gradients containing medium B and centrifuged in a Spinco SW 25.1 rotor at 22,500 rpm for 5 hr at 27–29°. The subunit fractions collected are indicated by the vertical bars.

The 40*S* fraction alone had little ability to catalyze the incorporation of phenylalanine from phenylalanine-tRNA into protein in the presence of polyuridylic acid, whereas the 60*S* fraction had approximately 20 per cent of the activity of the control (Table 1). The protein-synthetic activity of the 60*S* fraction was a direct reflection of the number of 80*S* particles present in it (Fig. 2). Reassociation of the combined subunits created particles that were active in polyuridylic acid-directed synthesis of polyphenylalanine. Indeed, the activity of recombinants exceeded that of control preparations, perhaps because of the presence in the latter of ribosomes containing endogenous mRNA that do not respond to polyuridylic acid.

Hybridization of ribosomal subunits: To determine whether subunits from normal and diabetic ribosomes differed in potential activity, we assayed polypeptide synthesis by hybrids containing a normal and a diabetic subunit. The necessity for added template RNA required the selection of suitable conditions for the assay. At magnesium concentrations (*ca.* 15 mM) giving maximal incorporation of phenylalanine in the presence of polyuridylic acid, ribosomes from diabetic animals were more active than those from normals (Table 1), perhaps because of the presence in diabetic preparations of a greater number of ribosomes lacking mRNA and therefore capable of responding to polyuridylic acid. However, at lower magnesium concentrations (*ca.* 9 mM), diabetic preparations were less responsive to polyuridylic acid than normal ribosomes (Table 1). For that rea-

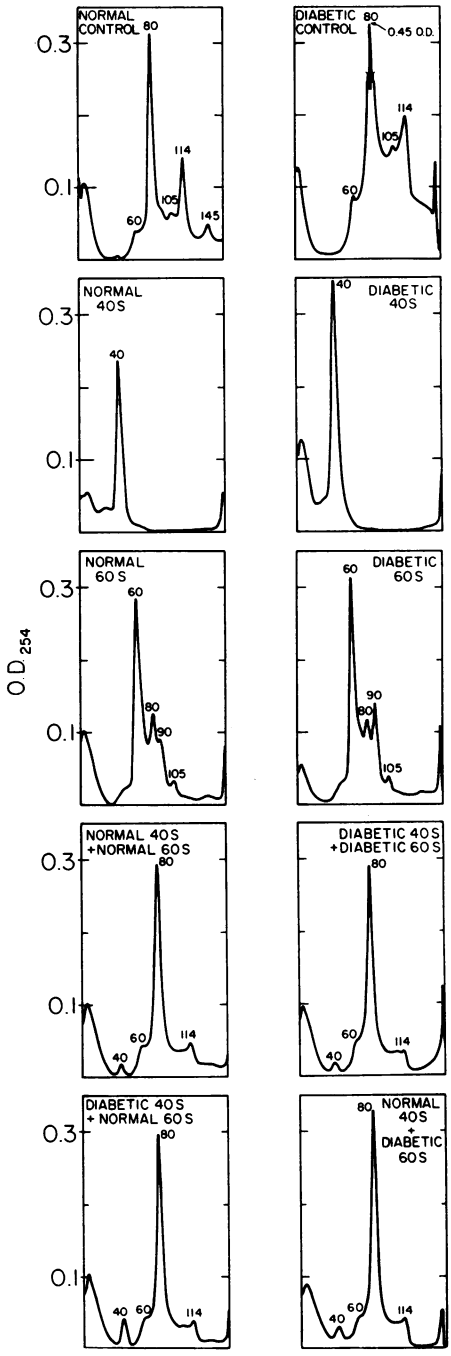

FIG. 2.—Formation of 80*S* particles from subunits of muscle ribosomes from normal and diabetic rats. After dialysis overnight against medium A, the ribosomal particles were layered in 15–30% linear sucrose gradients containing medium A and centrifuged in a Spinco SW 65 rotor at 60.000 rpm for 30 min at 28°.

TABLE 1. *The effect of magnesium concentration on protein synthesis by hybrids of ribosomal subunits from the muscle of normal and diabetic rats.*

Ribosomal particles	Stimulation of Incorporation by Polyuridylic Acid (Cpm/10 μg Ribosomal RNA)	
	9 mM Magnesium	15 mM Magnesium
Normal	2,702	5,831
Diabetic	1,928	11,402
Normal KCl-treated	1,318	5,756
Diabetic KCl-treated	718	7,513
Normal 60*S*	348	2,020
Diabetic 60*S*	178	1,370
Normal 40*S* + normal 60*S*	1,611	8,000
Diabetic 40*S* + diabetic 60*S*	816	7,710
Normal 40*S* + diabetic 60*S*	634	6,878
Diabetic 40*S* + normal 60*S*	1,722	8,444

The conditions for the assay of the incorporation into protein of radioactivity from H^3-phenylalanyl-tRNA (29,000 cpm/100 μg RNA) were as previously described,[3, 6] except that the reaction mixture contained 9 or 15 mM $MgCl_2$, as indicated, and approximately 1.5% sucrose. The reaction mixture (1 ml) contained 7.5–12.5 μg of ribosomal RNA and 100 μg of polyuridylic acid.

son, the activity of hybrid ribosomes was determined at both magnesium concentrations.

We prepared 40*S* and 60*S* subunits from ribosomes of normal and diabetic rats (as in Fig. 1), made hybrid combinations of the subunits, and measured their ability to catalyze polypeptide synthesis. When the four combinations of subunits were assayed at 15 mM magnesium (Table 1), they were found to possess similar activities; there was no clear evidence that either subunit from diabetic ribosomes was significantly different from its normal counterpart. A strikingly different result was given when protein synthesis was assayed at 9 mM magnesium (Table 1). At this concentration, the combinations that contained the diabetic 60*S* particle were much less efficient than those including the normal large subunit. The diabetic 40*S* subunit appeared to be equivalent to the normal 40*S* in potential activity (Table 1).

The extent of formation of hybrid 80S ribosomes: We considered that the lower activity of hybrids containing the diabetic 60*S* subunit might arise from a failure of this particle to associate efficiently with the 40*S* subunit to form the active 80*S* monoribosome. We therefore related the extent of the formation of 80*S* ribosomes to the ability of the reassociated or hybrid particles to synthesize protein (Fig. 2 and Table 2).

The dialyzed samples were analyzed on sucrose gradients, and unequivocal evidence of subunit reassociation was obtained (Fig. 2). The capacity of the reassociated particles to synthesize polyphenylalanine was closely related to the number of 80*S* ribosomes present (Table 2). It was also clear that subunits from normal and diabetic ribosomes could combine efficiently to form hybrid 80*S* particles; there was no indication that the diabetic 60*S* particle was less able to associate with 40*S* subunits than the normal 60*S* (Fig. 2). More significantly, the protein-synthetic activities of the recombinant 80*S* particles (cpm/80*S* ribosome) that contained the diabetic 60*S* subunit were found to be less than those containing the large subunit from normal animals (Table 2).

Since the reassociated particles do not catalyze amino acid incorporation in the

TABLE 2. *Protein synthesis by hybrid 80S particles formed from subunits of muscle ribosomes from normal and diabetic rats.*

Ribosomal particles	80S Particles* (%)	Incorporation (Cpm/10 μg Ribosomal RNA) Without polyuridylic acid	With polyuridylic acid	Stimulation	Stimulation/ 80S†
Normal KCl-treated	85	351	1,292	941	1,107
Diabetic KCl-treated	76	57	388	331	463
Normal 40S (N40)		0	100	100	
Diabetic 40S (D40)		0	4	4	
Normal 60S (N60)	19	7	347	340	1,789
Diabetic 60S (D60)	14	2	174	172	1,229
N40 + N60	79	10	1,370	1,360	1,722
D40 + D60	77	0	658	658	855
N40 + D60	75	0	654	654	872
D40 + N60	76	3	1,124	1,121	1,475

The conditions for the assay of the incorporation into protein of radioactivity from H^3-phenylalanyl-tRNA (30,000 cpm/100 μg RNA) were as previously described,[7, 6] except that the reaction mixture contained 9 mM $MgCl_2$, and approximately 1.5% sucrose. The reaction mixture (1 ml) contained 7.5–12.5 μg of ribosomal RNA, and the amount of polyuridylic acid, when present, was 100 μg.

* Includes dimers (114S) and trimers (145S); no significant amounts of larger polymers were present in these preparations.

† Determined from the proportion of 80S particles present.

absence of polyuridylic acid, it remains a possibility that additional factors are required for the binding and translation of natural mRNA.

Discussion.—The preparation of active subunits from mammalian ribosomes makes possible a variety of experiments that will aid in the analysis of the mechanism of protein synthesis in higher organisms. Unlike subunits prepared by removal of magnesium ions from ribosomes by treatment with ethylenediaminetetraacetate (EDTA) (refs. 9, 10; and Martin and Wool, unpublished results), 40S and 60S particles prepared by high concentrations of KCl readily reassociate to form active 80S monomers. The dissociation of ribosomes in high concentrations of monovalent cations is presumed to result from the displacement of divalent magnesium ions, which form ionic links between the subunits. The complete removal of magnesium ions by a chelating agent such as EDTA changes the subunits so that they sediment more slowly (approximately 30S and 50S) and become inactive; there is evidence, however, that subunits isolated from anterior pituitary ribosomes by treatment with EDTA do retain, at least in part, the ability to re-form active 80S particles (Adiga, Hussa, and Winnick, personal communication).

Reassociated ribosomes or hybrids containing the 60S subunit from the muscle of diabetic rats are less effective in polyuridylic acid-directed polypeptide synthesis at suboptimal magnesium concentrations. We do not know whether this defect in the 60S subunit is the cause or the effect of the decrease in protein synthesis in diabetic animals. At present, it is the only distinctive property of diabetic ribosomes that we know is retained by the subunits. Since it is a property of the 60S subunit, we may infer from bacterial studies[11, 12] that it is not the result of a defect in the binding sites for mRNA or aminoacyl tRNA, which are located on the small subunit. Binding of the nascent peptide and peptide bond

formation, however, appear to occur on the large subunit.[13] One or both of these functions may be altered in the ribosomes of diabetic animals, perhaps because of the inactivation or absence of some component of the subunit.

Summary.—40*S* and 60*S* subunits have been prepared from rat skeletal muscle ribosomes by treatment with KCl. The subunits are capable of reassociating to form 80*S* ribosomes active in polyuridylic acid-directed polypeptide synthesis. At suboptimal magnesium concentrations, ribosomes from normal rats synthesize more polypeptide in the presence of polyuridylic acid than ribosomes from diabetic animals. The difference in response is a property of the 60*S* subunit.

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