Aminoacyltransferase I-Catalysed Binding of Phenylalanyl-Transfer Ribonucleic Acid to Muscle Ribosomes from Normal and Diabetic Rats

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The aminoacyltransferase I-catalysed binding of phenylalanyl-tRNA (un-fractionated *Escherichia coli* B tRNA acylated with radioactive phenylalanine and 19 non-radioactive amino acids) to skeletal-muscle ribosomes from diabetic rats was less than that to ribosomes from normal rats when the Mg^{2+} concentration was low (7.5 mM); whereas just the reverse was true when the concentration of the cation was higher (15 mM). Thus the Mg^{2+} dependency of aminoacyltransferase I-catalysed binding of phenylalanyl-tRNA to ribosomes from normal and diabetic rats paralleled the effect of Mg^{2+} concentration on synthesis of polyphenylalanine reported before. During incubation at 7.5 mM- Mg^{2+} phenylalanyl-tRNA was bound only to ribosomes bearing nascent peptidyl-tRNA. There are fewer such ribosomes in a preparation from the muscle of diabetic animals because diabetic animals synthesize less protein *in vivo*. Thus the difference in polyphenylalanine synthesis *in vitro* is adequately explained by the difference in enzyme-catalysed binding of phenylalanyl-tRNA to ribosomes, however, the basis of the difference in protein synthesis *in vivo* is still unknown.

Preparations of ribosomes from the muscle of alloxan-diabetic rats contain a smaller number of polyribosomes (Stirewalt, Wool & Cavicchi, 1967) and are less active in protein synthesis than ribosomes from normal animals (Wool & Cavicchi, 1967). The administration of insulin to diabetic rats causes the reaggregation of the polyribosomes and restores the activity of the ribosomes even when RNA synthesis is inhibited by actinomycin (Wool & Cavicchi, 1966).

The decreased ability of ribosomes from diabetic rats for endogenous protein synthesis might then result from fewer ribosomes carrying template RNA. However, even after addition of an artificial template such as poly(U) or turnip-yellow-mosaic-virus RNA, ribosomes from diabetic rats still synthesize less protein (Wool *et al.* 1968; Rolleston, Wool & Martin, 1970), but the difference in synthesis of polyphenylalanine by ribosomes from normal and diabetic rats unlike the difference in endogenous protein synthesis, is critically dependent on the concentration of Mg^{2+} . Ribosomes from diabetic rats synthesize less polyphenylalanine than ribosomes from normal rats at lower concentrations of Mg^{2+} , but they actually synthesize more at higher concentrations of the cation (Wool *et al.* 1968). The greater polyphenylalanine

synthesis by ribosomes from diabetic rats at high Mg^{2+} concentration is probably the secondary consequence of their having less endogenous mRNA and as a result being able to bind more poly(U) in the favourable circumstances provided by the increased concentration of the bivalent cation; if ribosomes from diabetic rats bind more poly(U) they will, of course, synthesize more polyphenylalanine, all other things being equal. We have chosen to focus our attention on why ribosomes from diabetic rats synthesize less polyphenylalanine at low Mg^{2+} concentrations.

We have examined the effect of diabetes on individual steps in protein synthesis. The decreased activity of ribosomes from muscles of diabetic rats does not appear to be a reflexion of either decreased peptidyltransferase activity (Stirewalt & Wool, 1970) or a decreased ability to use aminoacyltransferase II (Leader, Wool & Leader, 1970). However, in assays without aminoacyltransferase I ribosomes from diabetic rats bound less phenylalanyl-tRNA (unfractionated *Escherichia coli* B tRNA acylated with radioactive phenylalanine and 19 non-radioactive amino acids) than those from normal rats at lowered concentrations of Mg^{2+} , whereas at high concentrations of Mg^{2+} just the reverse occurred (Wool

& Rolleston, 1971). Thus the binding of phenylalanyl-tRNA closely paralleled the translation of poly(U). It seemed that the diabetic defect in synthesis of polyphenylalanine might be the result of a lack of, or an inability to use, aminoacyl-transferase I.

The aim of this work was to decide between these two possibilities by comparing the ability of ribosomes from normal and diabetic rats to bind phenylalanyl-tRNA under the direction of poly(U) and in the presence of aminoacyltransferase I.

EXPERIMENTAL

General. The following have been described previously: the sources of material, the animals used (male Sprague-Dawley rats) and the method of inducing diabetes with alloxan (Wool & Cavicchi, 1967); the preparation of the ribosomal subunits and their reassociation (Martin & Wool, 1968; Martin, Rolleston, Low & Wool, 1969). Aminoacyl-tRNA was prepared as described by Wool & Cavicchi (1967), and passed through a column of Sephadex G-25 before use to remove contaminating nucleotides (Ravel, Mosteller & Hardesty, 1966). [^3H]Phenylalanine (5 Ci/mmol) and [^{14}C]phenylalanine (0.38 Ci/mmol) were purchased from New England Nuclear Corp., Boston, Mass., U.S.A., and diphenylalanine and triphenylalanine from Cyclo Chemical Corp., Los Angeles, Calif., U.S.A.

Preparation of aminoacyltransferase I. Aminoacyl-transferases I and II were prepared from rat liver 100 000g supernatant as described by Schneir & Moldave (1968). A typical separation of aminoacyltransferase I on

Sephadex G-200 is shown in Fig. 1. The aminoacyl-transferase I peak has almost no ability to catalyse polyphenylalanine synthesis in the absence of added transferase II, which would indicate it is virtually free of the latter.

Assay of binding of [^3H]phenylalanyl-tRNA. The procedure of Nirenberg & Leder (1964) was used and the assay conditions were chosen so that binding was directly proportional to the concentration of ribosomes. The complete reaction mixture (0.1 ml) contained in addition to ribosomes and [^3H]phenylalanyl-tRNA, 5 μmol of tris-HCl buffer, pH 7.5, 15 μmol of KCl, 0.05 μmol of GTP, 10 μg of poly(U), 0.5 μmol of ATP, 25 μg of aminoacyltransferase I and the amount of MgCl_2 indicated. Occasional omissions and variations are noted in the text. An assay was carried out as a routine with poly(U) omitted and this control value was subtracted from that for binding with poly(U). The reaction was started by the addition of the ribosomes (generally 7.5 μg of RNA) and incubation was for 30 min at 30°C. After incubation the tubes were placed in ice and diluted with 3 ml of ice-cold buffer containing 0.01 M-tris-HCl buffer, pH 7.5, 0.08 M-KCl and 0.01 M- MgCl_2 . The mixture was then poured quickly on to a nitrocellulose filter (Millipore filter; HAWP, 25 mm diam., 0.45 μm pore size) and washed as described by Nirenberg & Leder (1964). The filters were dried in glass vials, 10 ml of scintillation fluid [4 g of 2,5-diphenyloxazole and 0.2 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/l of toluene] were added and the radioactivities of the samples were counted in a Packard Tri-Carb scintillation spectrometer; the counting efficiency was 16%. Each 1000 c.p.m. was equivalent to 0.57 pmol of phenylalanine.

Fig. 1. Fractionation of aminoacyltransferase I on Sephadex G-200. A sample (approx. 25 mg of protein in 1.5 ml) of aminoacyltransferase I, partially purified on hydroxyapatite (Schneir & Moldave, 1968), was applied to a Sephadex G-200 column (75 cm $\times$ 2.5 cm) and eluted with 50 mM-tris-HCl buffer pH 7.2, 150 mM-KCl, 0.1 mM-EDTA and 1 mM- β -mercaptoethanol at a flow rate of 8 ml/h. Fractions (2.7 ml) were collected, their protein content determined from the extinction at 280 and 260 nm (Warburg & Christian, 1942), and 0.02 ml samples of selected fractions were assayed for their ability to support protein synthesis (Wool & Cavicchi, 1967) by washed liver ribosomes (Skogerson & Moldave, 1967). The assay was carried out with 30 μg of ribosomal RNA, 100 μg of [^3H]phenylalanyl-tRNA (containing 29 000 c.p.m.) 50 μg of poly(U) and in the presence and absence of 0.5 mg of aminoacyltransferase II. ▲, Incorporation (pmol) in the absence of aminoacyltransferase II; ●, incorporation (pmol) in the presence of aminoacyltransferase II; ----, protein (mg/ml). Fractions from the void volume (not shown) had no protein or enzyme activity.

Table 1. *Characteristics of the binding of [³H]-phenylalanyl-tRNA to muscle ribosomes*

Normal ribosomes (7.5 μ g of ribosomal RNA) were incubated with 80 μ g of [³H]phenylalanyl-tRNA (containing 52000 c.p.m.) in 10 mM-MgCl₂ in the reaction mixture described in the Experimental section except that no ATP was added and the other components were omitted where indicated. The results are corrected for radioactivity insoluble in hot trichloroacetic acid.

Incubation mixture	[³ H]Phenylalanyl-tRNA bound (pmol)
Complete system	2.47
Minus poly(U)	0.30
Minus aminoacyltransferase I	0.31
Minus GTP	0.29

A correction had to be made for a small amount of protein synthesis that occurred due to contamination of unwashed ribosomes with aminoacyltransferase II. This was done by measuring the hot trichloroacetic acid-insoluble radioactivity present after the incubation of separate identical assay mixtures (Wool & Cavicchi, 1967). This correction was generally about 20%.

The enzymic binding reaction was dependent on GTP, poly(U) and aminoacyltransferase I (Table 1). It must be emphasized that strict GTP dependence was not seen unless the aminoacyl-tRNA was passed through a Sephadex G-25 column and ATP was omitted from the assay.

Determination of the product formed by binding of [¹⁴C]phenylalanyl-tRNA to ribosomes. For determination of the product of binding of [¹⁴C]phenylalanyl-tRNA to ribosomes the volume of the reaction mixture was increased (to 1 ml or 3 ml) and 1 ml samples were layered on 4.3 ml of 10% sucrose in buffer (0.05 M-tris-HCl buffer, pH 7.5, 0.08 M-KCl and the concentration of MgCl₂ of the incubation mixture) and centrifuged at 60000 rev./min for 2.5 h at 4°C in a Spinco SW 65 rotor. The pellet was hydrolysed overnight with 0.3 M-KOH (0.2 ml) at room temperature, neutralized with 3 M-HCl and samples were taken to determine the recovery of protein and radioactivity. The remainder was applied to Whatmann 3MM filter paper and descending chromatography in butan-1-ol-ammonia-water (100:3:18, by vol.) was carried out (Pestka, 1968). Mono-, di- and tri-phenylalanine standards, run adjacent to the sample, were located with ninhydrin reagent. The chromatograms were cut into 1 cm strips and placed in glass bottles; 10 ml of scintillation fluid was added and the radioactivity measured with 60% efficiency.

RESULTS

Aminoacyltransferase I-catalysed binding of [³H]-phenylalanyl-tRNA to ribosomes from normal and diabetic rats. A comparison was made of enzymic and non-enzymic binding of [³H]phenylalanyl-tRNA to ribosomes from normal and diabetic rats at various Mg²⁺ concentrations in the presence of

Fig. 2. Effect of Mg²⁺ concentration on binding of [³H]phenylalanyl-tRNA to unwashed ribosomes in the presence of 5 mM-ATP. Ribosomes (7.5 μ g of ribosomal RNA) were incubated with 80 μ g of [³H]phenylalanyl-tRNA (containing 65000 c.p.m.) and various amounts of MgCl₂ in the reaction mixture described in the Experimental section except that aminoacyltransferase I was omitted where indicated. The values are corrected for radioactivity in hot trichloroacetic acid. ○, ●, Ribosomes from normal rats; △, ▲, ribosomes from diabetic rats. ●, ▲, Transferase I present; ○, △, transferase I absent.

5 mM-ATP (Fig. 2). At any concentration of Mg²⁺ up to 25 mM the amount of [³H]phenylalanyl-tRNA bound in the absence of enzyme was only a small fraction of that bound when the reaction was catalysed by aminoacyltransferase I. At a lower concentration of Mg²⁺ (7.5 mM) there was greater enzyme-catalysed binding to ribosomes from normal rats than to those from diabetic rats. However, at higher concentrations of Mg²⁺ (15 mM) the reverse was true. Thus the Mg²⁺ dependency of aminoacyltransferase I-catalysed binding of [³H]-phenylalanyl-tRNA to ribosomes from normal and diabetic rats paralleled the synthesis of poly-phenylalanine (Wool *et al.* 1968). Non-enzymic binding of phenylalanyl-tRNA to ribosomes from normal and diabetic rats had a very similar dependency on Mg²⁺ concentration even though it is not apparent in Fig. 2 (cf. Wool & Rolleston, 1971).

ATP is not required for the binding reaction but it was added to the assay mixture to make comparisons with earlier studies of protein synthesis easier. Qualitatively similar results for enzymic binding of phenylalanyl-tRNA were obtained when

ATP was omitted. The curves describing the binding of phenylalanyl-tRNA to ribosomes from normal and diabetic rats were shifted to a lower Mg^{2+} concentration, approx. 5mM lower, indicating that the effect of the ATP is mainly to chelate an approximately equimolar amount of Mg^{2+} (Korner, 1961).

If the difference in non-enzymic binding of aminoacyl-tRNA to ribosomes from normal and diabetic rats at low Mg^{2+} concentrations were due to differences in endogenous transferase I remaining on the ribosomes, then addition of saturating amounts of aminoacyltransferase I would be expected to abolish the difference. However, the decreased capacity of ribosomes from diabetic rats to bind aminoacyl-tRNA at lower concentrations of Mg^{2+} (7.5mM with 5mM-ATP) was manifest whatever the amount of aminoacyltransferase I added, even when the amount of enzyme seemed saturating.

Studies with washed muscle ribosomes. Because the ribosomes used to this point were contaminated with aminoacyltransferase II, it was necessary to apply a correction for the small amount of protein synthesis that occurred. Aminoacyltransferase II could be removed from muscle ribosomes, albeit with a decrease in protein synthetic ability, by the deoxycholate-potassium chloride wash method of McKeehan & Hardesty (1969). A comparison was made of the Mg^{2+} dependency of aminoacyltransferase I-catalysed binding of [3H]phenylalanyl-tRNA to ribosomes from normal and diabetic rats washed by their method (Fig. 3). As was the case with unwashed ribosomes there was more binding to the normal ribosomes at lower Mg^{2+} concentration (2.5 and 5mM, no ATP), but more to the ribosomes from diabetic rats at a higher concentration of the cation (10mM).

Analysis of the product formed as a result of binding of [^{14}C]phenylalanyl-tRNA to ribosomes. When the concentration of Mg^{2+} was 10mM (no ATP) 38% of the product of the aminoacyltransferase I-catalysed binding of [^{14}C]phenylalanyl-tRNA to deoxycholate-potassium chloride-washed normal ribosomes was monophenylalanine; the remainder was found at the origin. When the assay was carried out at 2.5mM- Mg^{2+} (no ATP) no monophenylalanine was recovered from ribosomes from either normal or diabetic rats. A small amount of radioactivity was spread diffusely throughout the chromatogram, but most of the radioactive material had remained at the origin (91 and 89% for ribosomes from normal and diabetic rats respectively) and the difference in amounts of this origin material was sufficient to account for the difference in phenylalanyl-tRNA bound to ribosomes from normal and diabetic rats at low concentration of Mg^{2+} .

Fig. 3. Effect of Mg^{2+} concentration on binding of [3H]phenylalanyl-tRNA to washed ribosomes from normal (●) and diabetic (▲) rats. Ribosomes (15 μ g of ribosomal RNA) were incubated with 80 μ g of [3H]phenylalanyl-tRNA (containing 56 000 c.p.m.) and various amounts of $MgCl_2$ in the reaction mixture described in the Experimental section except that ATP was omitted. There was no radioactivity insoluble in hot trichloroacetic acid.

DISCUSSION

The observation that the decreased capacity of ribosomes from diabetic rats to translate poly(U) at low Mg^{2+} concentrations is closely paralleled by a decrease in non-enzymic binding of phenylalanyl-tRNA (Wool & Rolleston, 1971) suggested that the diabetic defect might be the result of either a deficiency of aminoacyltransferase I or an inability to use the enzyme efficiently. The experiments described above were carried out to help decide between the two possibilities. For that purpose we studied the aminoacyltransferase I-catalysed binding of phenylalanyl-tRNA to ribosomes from normal and diabetic rats. At lower concentrations of Mg^{2+} ribosomes from diabetic rats bound less phenylalanyl-tRNA than normal, and the difference persisted even with amounts of aminoacyltransferase I that seemed saturating. The results then suggested that ribosomes from diabetic rats could not use aminoacyltransferase I effectively.

The exact functional significance of binding of aminoacyl-tRNA to ribosomes can only be accurately assessed when the product of the reaction is

known. At high Mg^{2+} concentrations a substantial portion of the product of the binding was monophenylalanine. Other workers have reported that monophenylalanine is the only product of enzymic binding to transferase II-free animal ribosomes (Lin, McKeehan, Culp & Hardesty, 1969; Siler & Moldave, 1969). However, in our experiments the crucial observation was that at lower concentrations of Mg^{2+} (2.5 mM with no ATP), the concentration of the cation at which the diabetic defect in binding of aminoacyl-tRNA was seen, almost all of the radioactive material bound to the ribosome remained at the origin when chromatographed on paper: no monophenylalanine was recovered. We have shown that the 'origin material' is a peptide containing a single covalently bound C-terminal [^{14}C]phenylalanine and that the source of the peptide is peptidyl-tRNA (Castles, Rolleston & Wool, 1971). It would appear, therefore, that at low concentrations of Mg^{2+} phenylalanyl-tRNA is bound only to ribosomes that carry peptidyl-tRNA; that in the strictest sense no initiation of synthesis of new peptide chains occurs with transferase I. This conclusion is supported by the observations that puromycin treatment of the ribosomes causes a loss of their ability for non-enzymic (Castles *et al.* 1971) or enzymic binding (results not shown) at low Mg^{2+} concentrations. Moreover, Shafritz & Anderson (1970) have shown that initiation factors, distinct from transferase I and II, are required for polyphenylalanine synthesis by reticulocyte ribosomes at low Mg^{2+} concentrations.

The decrease in enzymic binding of phenylalanyl-tRNA to ribosomes from diabetic rats seems a consequence of decreased endogenous peptidyl-tRNA on the particles. This, of course, is a reflexion of the decreased protein synthesis occurring on ribosomes from diabetic rats before they are isolated. Thus, we have a satisfactory explanation for the decreased translation of poly(U) *in vitro*, but cannot account for the decreased protein synthesis *in vivo*. Indeed, it now appears unlikely that there is any difference in the ability of stripped ribosomes from normal and diabetic rats to catalyse the elongation reactions of protein synthesis. We must therefore consider the possibility that the difference in protein synthesis seen *in vivo* reflects a

defect in the capacity of muscle from diabetic rats to initiate protein synthesis.

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REFERENCES

- Castles, J. J., Rolleston, F. S. & Wool, I. G. (1971). *J. biol. Chem.* **246**, 1799.
- Korner, A. (1961). *Biochem. J.* **81**, 168.
- Leader, D. P., Wool, I. G. & Leader, J. E. (1970). *Acta diabet. lat.* **7**, 990.
- Lin, S.-Y., McKeehan, W. L., Culp, W. & Hardesty, B. (1969). *J. biol. Chem.* **244**, 4340.
- McKeehan, W. L. & Hardesty, B. (1969). *J. biol. Chem.* **244**, 4330.
- Martin, T. E., Rolleston, F. S., Low, R. B. & Wool, I. G. (1969). *J. molec. Biol.* **43**, 135.
- Martin, T. E. & Wool, I. G. (1968). *Proc. natn. Acad. Sci. U.S.A.* **60**, 569.
- Nirenberg, M. & Leder, P. (1964). *Science, N.Y.*, **145**, 1399.
- Pestka, S. (1968). *J. biol. Chem.* **243**, 2810.
- Ravel, J. M., Mosteller, R. D. & Hardesty, B. (1966). *Proc. natn. Acad. Sci. U.S.A.* **56**, 701.
- Rolleston, F. S., Wool, I. G. & Martin, T. E. (1970). *Biochem. J.* **117**, 899.
- Schneir, M. & Moldave, K. (1968). *Biochim. biophys. Acta*, **166**, 58.
- Shafritz, D. A. & Anderson, W. F. (1970). *J. biol. Chem.* **245**, 5553.
- Siler, J. & Moldave, K. (1969). *Biochim. biophys. Acta*, **195**, 123.
- Skogerson, L. & Moldave, K. (1967). *Biochem. biophys. Res. Commun.* **27**, 568.
- Stirewalt, W. S. & Wool, I. G. (1970). *FEBS Lett.* **10**, 38.
- Stirewalt, W. S., Wool, I. G. & Cavicchi, P. (1967). *Proc. natn. Acad. Sci. U.S.A.* **57**, 1885.
- Warburg, O. & Christian, W. (1942). *Biochem. Z.* **310**, 384.
- Wool, I. G. & Cavicchi, P. (1966). *Proc. natn. Acad. Sci. U.S.A.* **56**, 991.
- Wool, I. G. & Cavicchi, P. (1967). *Biochemistry, Easton*, **6**, 1231.
- Wool, I. G. & Rolleston, F. S. (1971). In *The Action of Hormones—Genes to Population*, p. 54. Ed. by Foa, P. P. Fort Lauderdale: C. C. Thomas.
- Wool, I. G., Stirewalt, W. S., Kurihara, K., Low, R. B., Bailey, P. & Oyer, D. (1968). *Recent Prog. Horm. Res.* **24**, 139.