Influence of Cell Disruption Methods on Polyuridylic Acid Dependent Polyphenylalanine Synthesis by Isolated Ribosomes of Mycobacteria

In previous papers we described the isolation and identification of mycobacterial ribosomes and evaluated some of the variables in a test system for demonstration of their polyphenylalanine synthesizing activity in vitro[1,2]. From these experiments it became evident that care must be exercised in the conditions for disruption of cells, because some factors of the disruption techniques may exert damaging effects on the biological activity of isolated intracellular components. The present paper describes a comparison of the 2 most commonly employed disruption techniques for their effectiveness in preparing biologically active subcellular fractions of mycobacteria.

Mycobacterium bovis (BCG) cultivated as a pellicle on Sauton medium was harvested, washed, frozen and stored at − 20 °C. Equal portions of the bacterial mass was either ground with alumina powder (Norton abrasives, Worcester, Mass.) in a pre-chilled mortar at 4 °C for 10 min (fractions designated 'A') or for 25 min (fractions designated 'B') or were disrupted in a Ribi Refrigerated Cell Fractionator (Model RF-1, Ivan Sorvall Inc., Norwalk, Conn.) at 20,000 ψ at temperatures ranging from − 5 to 4 °C fractions designated 'R'). Ribosomes and supernatant (S-105) fractions were isolated from all portions following procedures described previously[1,2]. The peptide synthesizing capacity of the isolated ribosomes was compared in a system detailed previously[2] using polyuridylic acid as an artificial messenger RNA coding for polyphenylalanine. Studies were made both with homologous test systems (in which ribosomes and S-105 fractions came from the same cell disruption procedures) and with heterologous systems (ribosomes and S-105 fractions from different disruption procedures). The radioactivity resulting from the incorporation of ^{14}C-1-phenylalanine into the hot trichloracetic acid (TCA) insoluble fraction was measured by the liquid scintillation method. In 1 experiment a comparison was made between the synthesizing capacity of freshly isolated fractions and those stored for 24 h in a buffer containing 0.006 *M* β-mercaptoethanol at 4 °C.

The results are summarized in the Table. No significant differences were observed in polyphenylalanine synthesizing activity between homologous systems, prepared from BCG cells, harvested after 11 days (experiment No. 1) or 18 days of cultivation (experiment No. 2). Age of the cells used was probably the main reason for differences in the activity of subcellular fractions between these 2 experiments. This is in accord with a previous observation[2]. Considering the heterologous systems it can be seen that the combination of ribosomes disrupted in the pressure cell with S-105 fraction from BCG ground with alumina in a mortar led in both experiments to a significant enhancement of the incorporation rate. The opposite combination (Rib$_A$ + S − 105$_R$), on the other hand, exhibited a significantly reduced rate of incorporation. The activity of fractions resulting from grinding in a mortar for 10 min did not differ from that obtained grinding for 25 min. Storage for 24 h was followed by decreased activity, as was reported previously[2], but the above mentioned differences in activity of heterologous systems were maintained.

The essential differences in the disruption techniques is that one consists of exposure of a bacterial mass to high pressure (20,000 ψ = 1,361 atm), followed by slow release through a needle valve at controlled temperatures, while the other employs grinding in a chilled mortar with alumina. One interpretation of the results of these experiments is that each technique may exert a harmful effect on a particular target structure. Conditions in the pressure cell may have affected components of the S-105 fraction containing mainly activating enzymes. A considerable amount of work has been done on the effect of pressure on the action of a number of intracellular enzymes[3]. Values above 500 atm exerted structural and functional changes in protein molecules and damage of proteosynthesizing polysomal system[4–7]. Our experiments failed to prove evidence of alteration of ribosomes in the pressure cell in a way that would impair the binding of polyU. The low values for endogenous incorporation of ^{14}C-1-phenylalanine preclude drawing conclusions of the high pressure effect on natural messenger RNA.

Grinding frozen cells with abrasive particles may be followed by damage to the integrity of polysomal complexes. We have observed that intensive grinding of cells leads to reduction of 70S ribosomal monomers and to an

Incorporation of ^{14}C-1-phenylalanine into hot TCA insoluble peptides on ribosomes isolated from BCG using different disruption techniques

System	Fresh prepared		Stored 24 h at 4 °C	
	Complete	Without polyU	Complete	Without polyU
Experiment No. 1				
Homologous				
Rib$_A$ + S105$_A$	8289 ± 1969	99	2203 ± 629	89
Rib$_R$ + S105$_R$	6318 ± 366	65	2419 ± 1502	89
Heterologous				
Rib$_A$ + S105$_R$			759 ± 132	59
Rib$_R$ + S105$_A$			7106 ± 1177	103
Experiment No. 2				
Homologous				
Rib$_A$ + S105$_A$	133 ± 41	74		
Rib$_B$ + S105$_B$	159 ± 31	75		
Rib$_R$ + S105$_R$	663 ± 228	36		
Heterologous				
Rib$_A$ + S105$_R$	117 ± 32	35		
Rib$_R$ + S105$_A$	791 ± 28	46		
Rib$_B$ + S105$_R$	78 ± 17	70		
Rib$_R$ + S105$_B$	832 ± 226	70		

All values are cpm/mg of ribosomal protein (with standard deviation). Rib, ribosomal fraction (pellet after 105,000 *g* centrifugation washed twice). S-105, supernatant fraction after isolation of ribosomal fraction. Subscript -A, fractions prepared from BCG disrupted by grinding in a chilled mortar for 10 min. Subscript -B, fractions prepared from BCG disrupted by grinding in a chilled mortar for 25 min. Subscript -R, fractions prepared from BCG disrupted in a Ribi Cell fractionator.

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increase of 30S and 50S subunits as evidenced by sucrose density gradients and analytical ultracentrifugation studies. In the present experiments we have noted a significant decrease of activity in incorporation systems composed of ribosomes derived from cells ground with alumina and S-105 fractions from cells disrupted in the pressure cell. The possibility of ribosomal impairment by sharp abrasives was already stressed as a disadvantage of the otherwise simple and convenient method of bacterial cells disruption in a mortar[8].

In our experiments these destructive influences were of limited extent, so that the effects were masked in homologous systems (taking into account standard deviations from replicate samples). These destructive effects, however, may be particularly important in detailed functional studies of enzyme systems at the subcellular level.

Zusammenfassung. Der Einfluss verschiedener Desintegrierungsmethoden von Tuberkelbazillen auf die biologische Aktivität von isolierten subzellulären Fraktionen wird beschrieben.

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Mangelnde Reaktivierung reduzierter RNase durch Zajdela-Hepatom-Ascites-Zellen

Unter geeigneten Bedingungen können aktive Enzyme in inaktive Proteine umgewandelt und anschliessend wieder in ihre ursprüngliche Form regeneriert werden. Es wurde gezeigt[1,2], dass nach Reduktion der Disulfid-Brücken und Auflösung der tertiären Struktur der RNase die Reoxydation unter Wiedererlangung der biologischen Aktivität möglich ist. VENETIANER und STRAUB[3] wiesen in Tauben- und Hühnerpankreas und GOLDBERGER et al.[4] in Rattenlebermikrosomen ein Enzym nach, das den Prozess dieser Reaktivierung beschleunigt. Ausser den Mikrosomen werden noch Faktoren aus dem Überstand benötigt, die keine Proteine sind.

Es erschien daher interessant zu untersuchen, ob die Reaktivierung auch bei Hepatomen möglich ist. Dabei wurde das Verhalten von Hepatom-Ascites-Zellen auf die Fähigkeit zur Umwandlung einer inaktiven ungeordneten RNase in eine aktive Form geprüft. Die zur Enzymreaktion verwendeten Zellfraktionen wurden nach GOLDBERGER et al.[4] hergestellt (Modifikationen: Durchspülung der Lebern, Homogenisierung im Messer-Bühler-Homogenisator). Die RNase (Boehringer p.A.) wurde in Analogie zu ANFINSEN et al.[2] reduziert. Die RNase-Bestimmung erfolgte bei pH 5,0 nach SPAHR und HOLLINGWORTH[5].

Die Zajdela-Hepatom-Zellen waren auf Mäuse transplantiert. Die Normalleber stammt von Sprague-Dawley-Ratten. Das Reaktionsgemisch bestand nach GOLDBERGER et al.[4] aus reduzierter RNase 0,010 mg, in 0,05 *M Tris* Puffer pH 7,4 Mikrosomenfraktion 8,25 mg Proteingehalt und Überstand 23,6 mg Protein, gemessen nach LOWRY et al.[6] im Gemisch. Endvolumen 5,5 ml, welches bei 37 °C 20 min inkubiert wurde. Es wurde jeweils gegen einen Leerwert reduzierter RNase in gleicher Konzentration, in Puffer gelöst, gemessen. Angegeben sind Mittelwerte aus 3 Bestimmungen mit Maximalabweichung. Die in der Tabelle angegebenen Werte beziehen sich auf die Extinktionsdifferenz (bei 260 μm) der RNase-Bestimmung vor und nach der Inkubation des Reaktivierungsgemisches.

Da das Hämoglobin eine reaktivierende Wirkung besitzt[7], wurden die Lebern vor Entnahme über die Pfortader durchspült. In unseren Untersuchungen konnten wir die Reaktivierungsfähigkeit der Leber bestätigen. Dabei zeigten sich keine Geschlechts- und Stammesunterschiede. Durch Zajdela-Hepatom-Fraktionen liess sich nur eine geringfügige Reaktivierung reduzierter RNase nachweisen, gleichgültig, ob die Hepatomzellen auf Ratten oder Mäuse (über Hamster) transplantiert worden waren.

Weitere Untersuchungen müssen zeigen, ob es sich hier um einen spezifischen Unterschied zwischen Normal- und Tumorzellen handelt, zumal bei frühem embryonalem Gewebe ebenfalls keine Aktivität gefunden wurde[7]. Möglicherweise ist ausser der RNase-Aktivierung auch die Aktivierung anderer Enzyme im Tumor beeinträchtigt.

Summary. Decreased reactivation of inactivated RNase by microsomes from a hepatoma as compared to normal liver was observed and is described in more detail. This hampered reactivation of enzymes in general might be responsible for other enzyme deletions in tumours.

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Zajdela-Hepatom		Normalleber	
Experiment Nr.	Extinktionsdifferenz	Experiment Nr.	Extinktionsdifferenz
1	0,069 ± 0,005	1	0,198 ± 0,003
2	0,006 ± 0	2	0,288 ± 0,010
3	0,019 ± 0,008	3	0,236 ± 0,002
4	0,031 ± 0,006	4	0,275 ± 0,007
5	0,055 ± 0,003	5	0,323 ± 0,005
6	0,062 ± 0,005	6	0,208 ± 0,011

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