The Distribution of α -Amylase-Forming Ability between the Membrane and Soluble Fractions of a Cell-Free Preparation of *Bacillus amyloliquefaciens*

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Interest has been maintained over the years in the mode of formation and secretion of extracellular enzymes by bacteria. However, studies with intact bacterial cells have not met with a great deal of success in elucidating details of this process (Lampen, 1965).

A novel approach to the problem has recently been adopted, namely to investigate protein synthesis at the subcellular level in the exoenzyme-secreting organism *Bacillus amyloliquefaciens* in an attempt to localize the site of α -amylase (EC 3.2.1.1) formation. A study had already been made of the conditions of cell disruption that best preserve the protein-synthesizing machinery, the effects of Mg^{2+} and K^+ concentrations on polyribosome structure and function in cell-free extracts and the influence of ionic conditions on the distribution of polyribosomes between soluble and insoluble membrane fractions in *B. amyloliquefaciens* cell lysates (Coleman, 1969*a,b,c*). The present communication describes a comparative study of the abilities of membrane and soluble fractions of lysozyme lysates of cells actively secreting exoenzyme to incorporate radioactive amino acids into protein and form α -amylase by an energy-dependent process under conditions that have been found to be optimum for protein synthesis in this system.

A 100 ml portion of a 26 h culture of post-exponential-phase cells of *B. amyloliquefaciens* grown in a complex medium (Coleman, 1967, 1969*b*) was centrifuged, the supernatant fraction was discarded and the cell pellet was frozen in liquid nitrogen. The frozen pellet was then thawed in ice-cold 50 mM-tris-HCl buffer, pH 7.6, containing 10 mM- Mg^{2+} and 85 mM- K^+ and washed three times by centrifuging and resuspending. The washed cells were finally resuspended to 10 ml in washing buffer, and 5 ml of the suspension was retained. The remaining 5 ml of cell suspension was treated with lysozyme as described by Coleman (1969*b*) and the resulting lysate, which under the electron microscope showed no evidence of intact cells, was centrifuged at 20000*g* for 10 min at 0°C. The supernatant fraction was retained as a source of soluble polyribosomes, and the pellet was resuspended to the original volume in 50 mM-tris-HCl buffer, pH 7.6, containing 10 mM- Mg^{2+} and 85 mM- K^+ and

retained as a source of membrane-bound polyribosomes. The washed-cell suspension, membrane and soluble fractions were then included in cell-free protein-synthesizing systems and their abilities to incorporate ^{14}C -labelled amino acids into protein and to form α -amylase were studied.

The results (Table 1) show that frozen, thawed and washed cells were capable of supporting α -amylase formation and incorporation of ^{14}C -labelled L-amino acids at levels of 5 and 15% respectively of the amounts formed in a cell lysate. Since no more than a trace amount of whole cells was present in the membrane fraction of the lysate any contribution by it to the observed activity of the membrane-bound ribosomes would be negligible. Also, the specific radioactivities of the membrane and soluble fractions were closely similar, showing the ability of the two fractions to incorporate amino acids was proportional to their rRNA contents. However, the distribution of α -amylase-forming ability was quite different, 40% of that formed during the incubation being produced by the membrane fraction, which contained only 11% of the rRNA; thus the amount of α -amylase produced per unit of RNA by the membrane fraction was more than five times that formed in the soluble fraction.

A number of models can be proposed on the basis of these results to describe the site of α -amylase formation. In each case it is assumed, consistently with the experimental findings, that during the incubations polypeptide chains are not synthesized *de novo* but rather partially formed polypeptide chains are extended such that the longest ones are completed.

The first model is based on the idea that, whereas there is a similar distribution of all classes of ribosomal material in both the membrane and soluble fractions, there is also a specific attachment of polyribosomes with longer peptide chains, nearing completion, at the membrane (Aronson, 1966), with polyribosomes bearing shorter peptide chains free in the cytoplasm. This is possible if mRNA molecules are not saturated with ribosomes, i.e. if mRNA molecules are not occupied by ribosomes packed as closely together as possible over their whole lengths. The extreme example is that of a

Table 1. *Energy-dependent increases in α -amylase activity and incorporation of ^{14}C -labelled L-amino acids*

Incorporation of ^{14}C -labelled L-amino acids was studied in the system described by Coleman (1969d) containing 10 mM- Mg^{2+} and 85 mM- K^+ , in the presence and in the absence of ATP plus an ATP-generating system. α -Amylase formation was studied in a similar system, the scale of which was increased fourfold and the radioactive amino acid mixture was replaced by a mixture of equal amounts of non-radioactive amino acids at a concentration ten times that of the ^{14}C -labelled L-amino acids included in the radioactivity-incorporation experiment. Incubations were carried out for 30 min at 30°C . α -Amylase was determined by a modification of the method described by Coleman & Elliott (1962), which increased the sensitivity 100-fold. In the incubation containing the soluble fraction as a source of polyribosomes a sample of the whole reaction mixture was assayed, whereas the reaction mixtures containing whole cells and membrane fraction were centrifuged at the end of the incubation and the α -amylase contents of the supernatant fractions were determined.

Preparation	Increase in (α -amylase activity (units/mg of RNA)	Increase in ^{14}C -labelled L-amino acid incorporation (c.p.m./mg of RNA)
Frozen and thawed cells before lysis	0.04	778
Frozen and thawed cells after lysis*	0.73 (0.29 membrane, 0.44 soluble)	5094 (590 membrane, 4504 soluble)
Membrane fraction alone	2.62	5268
Soluble fraction alone	0.49	5027

* α -Amylase activity and incorporating ability of the cell lysate were taken as the sums of the individual values for membrane and soluble fractions from an amount of cell lysate containing a total of 1 mg of rRNA.

monoribosome, a single ribosome attached to a mRNA molecule as an independent species, such that on initiation of a new peptide chain the whole complex is free in the cytoplasm, whereas when the single growing peptide chain is approaching completion the monoribosome becomes membrane-bound owing to the presence of this large though yet incomplete polypeptide. If all partially formed chains are extended by a similar number of amino acid residues, the specific radioactivities for membrane-bound and soluble ribosomes will be the same. However, this extension will result in the completion of only the longer polypeptide chains, i.e. those in the membrane fraction but not those in the soluble fraction. The appearance of α -amylase in both fractions is inconsistent with the above, but might be explained in terms of a release of 60% of 'long-chain peptide' polyribosomes from the membrane on lysis caused by the overriding effect of a change in ionic environment as, for example, might be expected if the $\text{Mg}^{2+}/\text{K}^+$ concentration ratio of the lysing buffer were lower than in the cytoplasm of the cell (Coleman, 1969c).

A second model, similar to the first, may be described differing in having equal distribution of all partially formed polypeptide chain sizes in the membrane and soluble fractions and a concentration of exoenzyme-forming polyribosomes compared with general cell-protein polyribosomes at the membrane. The selection mechanism is not specified, but it is possible that a distinction could

be made between the longer partially formed polypeptide chains on the basis of their ability to form disulphide bridges; exoenzymes contain no cysteine and are thus unable to form such bonds (Pollock & Richmond, 1962). In this case specific radioactivities of membrane-bound and soluble ribosomes could be the same and as in the first model there should be no α -amylase formation in the soluble fraction. However, its appearance in the soluble fraction might again be explained in terms of lowering of the $\text{Mg}^{2+}/\text{K}^+$ concentration ratio on lysis causing a liberation of 60% of the membrane-bound ribosomes. This model fits the results except for explaining the binding of ribosomal subunits to the membrane; this, however, might also be an artifact of conditions of lysis.

A third model is possible in which there is no redistribution of ribosomal material between membrane and soluble fractions on lysis as might be observed if the $\text{Mg}^{2+}/\text{K}^+$ concentration ratio of the lysing buffer were the same as that in the cytoplasm of the intact cell. In this case the ability of membrane-bound and soluble polyribosomes to synthesize α -amylase is considered to be directly proportional to their radioactive amino acid-incorporating abilities, in which case 80% of the fivefold excess of production of exoenzyme in the membrane fraction would represent energy-dependent liberation of preformed material. If, however, this were the correct model then one perhaps might expect a bigger energy-dependent increase in

α -amylase formation by the whole cells than was observed.

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