Detection of Inactive Enzyme Molecules in Ageing Organisms

by

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The synthesis and accumulation of non-functional protein molecules may play a significant part in senescence and the eventual death of organisms. In this communication we report the use of immunological techniques to look directly for the presence of catalytically altered or inactive enzyme molecules in ageing nematodes. The immunological approach is both specific and sensitive and allows for the expression of results in units of catalytic activity per unit of antigenic activity, thus allowing for the detection of partially or totally inactive enzyme molecules present in the form of cross reacting material (CRM). This approach has been successfully used in detecting cross reacting protein in bacterial and fungal mutants lacking specific enzyme activities arising from single amino-acid changes caused by point mutations.

Although the recent observations by Smith and Marcker suggest that the formylatable form of the cytoplasmic eukaryote Met-tRNA may indeed be the initiating tRNA for eukaryotic protein synthesis, our experiments show both formylatable and non-formylatable cytoplasmic Met-tRNAs of yeast to react equally well with our T fractions.

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11. Lipmann, F., In The Role of Nucleotides for the Function and Conformation of Enzymes, 195 (Munksgaard, Copenhagen, 1960).

We chose the nematode Turbatrix aceti as a model organism, which has proved suitable for research on ageing for the following reasons: its life span is short—a mean of 25-30 days; it has a fixed number of cells after hatching, with the exception of the reproductive system; it lacks regenerative capacity; it possesses a relatively small number of cell types and exhibits full differentiation at the time of hatching; and it can be raised in axenic conditions in quantities suitable for biochemical studies.

Previous studies have shown that the specific activities of several enzymes in T. aceti show a general decline with age. It was not known, however, whether this decline in activity was the result of a reduction in the number of enzyme molecules per cell or of the progressive formation or accumulation of inactive molecules. The enzyme carriers test with reasonably pure tRNA fractions. The separation of the yeast Met-tRNAs was done as outlined by Henes et al. for E. coli Met-tRNA. This method fractionates the phenacyl-acetylated methionyl-tRNAs from yeast on OD-cellulose at 4° C. One fraction only of the two species was formylatable with the E. coli transformylase and folic acid (see legend to Fig. 3). The isolated non-formylatable Met-tRNA was and still contained 10 per cent tRNA which does not affect this conclusion for, if it were rejected and only Met-tRNA was bound, the expected slope would be 0-1. The slope of 0-0 found experimentally just confirms that both formylatable and non-formylatable factors form a ternary complex, as might be expected from the results of Fig. 2 where the natural mixture of both shows a slope of 1-0 by contrast with a slope of 0-6 with the mixture of E. coli Met-tRNAs. In other experiments (not included) the purified Met-tRNA was bound, the expected slope would be 0-1. Blocking the amino group of the yeast Met-tRNA by formylation, however, caused rejections by the Factor (Fig. 3). Similarly it was found (not shown) that acetylation of the amino group of yeast Met-tRNA by the method of Haendl and Chapeville prevents formation of a ternary complex with yeast T factor. On the other hand, for pure Met-tRNA of E. coli, we confirm that it is not bound to T factor. This indicates that in Fig. 2 the 40 per cent of E. coli tRNA charged with methionine that did not bind was indeed the Met-tRNA.
chosen for our present studies, isocitrate lyase, also showed a decline in specific activity with age (see below). This enzyme was presumed to be a good immunogen because it is not present in higher organisms and has been reported to have a high molecular weight in both Chlorella pyrenoidosa and Pseudomonas indigofera. Moreover, this enzyme has been shown to consist of a single molecular species in certain other experimental systems.

Turbinaria aceti were grown in sterile axenic culture conditions as described by Rothstein and Cook. Populations of T. aceti of uniform age were grown and, at various times after the initiation of culture, populations of uniform age were harvested, thoroughly cleaned of medium components, lyophilized and stored at -20°C until used for enzyme analysis.

Lyophilized populations of T. aceti of uniform ages were suspended in cold 0.32 M sodium phosphate buffer containing 5 mM EDTA and 0.5 mM dithiothreitol. Each suspension was homogenized at 400 atmospheres in a cold French pressure cell. The homogenate was incubated at 4°C for 10 min with 0.5 per cent `Sarkosyl' and subsequently centrifuged at 36,000 g for 30 min. The pellet was discarded and the supernatant, referred to as crude enzyme, was maintained at 4°C for enzyme assay.

Synchronized populations of T. aceti ranging in age from day 0 to day 45 were assayed for isocitrate lyase activity. For most age points, several determinations were made on two or more independently grown populations. The specific activity of isocitrate lyase in T. aceti starts at a high level in early life and gradually decreases with increasing age (Fig. 1). This decrease in enzyme activity could either reflect a true reflexion of the amount of enzyme molecules present in the ageing organism or be attributed in whole or in part to the production and/or accumulation of molecules with impaired enzymatic function. To distinguish between these two alternatives the following series of experiments were performed. Antibody to the crude enzyme preparation of 5-day-old T. aceti was prepared by immunizing a rabbit by repeated intradermal and intramuscular injections of the crude enzyme preparation in emulsion with an equal volume of Freund's complete adjuvant.

Before the commencement of the immunization schedule the rabbit was bled for normal serum. The effect of antibody on enzyme activity was measured by mixing various amounts of antibody, normal rabbit serum or phosphate buffer containing EDTA and dithiothreitol with crude enzyme preparations of synchronized populations of various ages. At the onset of each individual experiment all crude enzyme preparations were adjusted to the same initial activity. Crude enzyme preparations of each age were divided into three aliquots, one made 8.75 per cent with anti-serum, the second made 8.75 per cent with normal rabbit serum and the third a control of equal volume and initial enzyme activity in buffer alone. The amount of antibody added was predetermined to allow all samples to fall within the sharply ascending antigen excess part of the precipitation curve in order to increase the sensitivity of the assay and allow for observation of differences in precipitation of the enzyme in different age samples. It was found that normal rabbit serum had no effect on enzyme activity and gave the same results as the enzyme in buffer controls. The activity of the enzyme was measured immediately after addition of antibody and in certain experiments once again after 1 h, and 24 h incubation at 4°C. It was found that there was a certain degree of inactivation of the enzyme on initial contact with antibody. No such inactivation was noted in the normal rabbit serum controls. Subsequent to this inactivation of initial contact with antibody, the activity of the enzyme remained stable at 4°C. The antibody-homogenate mixtures as well as normal rabbit serum and buffer controls were incubated overnight at 4°C and then centrifuged at 12,000g for 15 min to remove any antigen-antibody precipitate that might have formed. The resulting supernatant was then assayed for residual enzyme activity.

As the populations increase in age, less enzyme is precipitated per fixed amount of antibody (Fig. 2). Thus, for the same level of enzyme activity, crude enzyme preparations from old animals contain more antigenic material than is detected in young animals. Because it has been described in certain other systems and will be demonstrated for this system that isocitrate lyase consists of a single molecular species the results depicted in Fig. 2 may be interpreted as follows: the increase in detectable antigenic material in ageing populations may be attributed to the reduced activity of all the enzyme molecules present or to the presence of some enzyme molecules with a normal degree of activity and others totally lacking enzymatic function.

If the amount of antisemur is increased it is possible to precipitate comparable proportions of enzyme activity...
from populations of 5-day and 35-day-old animals (Fig. 3). The precipitation curves of enzyme from 5 and 35-day-old animals demonstrate the identity of the enzyme in both preparations, for they exhibit the same slope and degree of maximal per cent precipitation. The 35-day preparation is shifted to the region of higher antibody concentration, indicating the presence of a considerably higher concentration of enzyme antigen per unit of enzymatic activity than is present on day 5.

To establish whether the difference in enzyme activity per unit of antigen may arise from the presence of an inhibitor in older populations, crude enzyme preparations of young and old animals were mixed together in equal volumes and tested immediately after mixing as well as after incubation overnight at 4°C. These experiments failed to demonstrate the presence of an inhibitor of enzyme activity in homogenates of ageing populations.

These experiments indicate either that the isocitrate lyase present in ageing populations of *T. aceti* is composed of enzyme molecules with lower activity than that present in young adults or that the older organisms contain two populations of enzyme molecules, one with completely normal activity—like that of the young adults—and the other, totally inactive molecules which can be detected only as cross-reacting material recognized by antibody. Two lines of evidence make the latter situation the more probable. When the antibody and enzyme interact, some of the enzyme is inactivated, because of the binding of antibody with regions of the enzyme molecule in or very near to the active site of the enzyme.

If the properties of the area of the active site are the same for all the enzymatically active isocitrate lyase molecules of old as well as young animals, then they ought to be inactivated to the same degree by increasing amounts of antibody. If, on the other hand, the entire population of enzyme molecules bear altered active sites, one ought to see differing inactivation curves when enzyme activity is kept constant and the amount of antibody is varied. In Fig. 4 it is clear that the inactivation of enzyme by increasing amounts of antibody is the same for the enzyme of 5 and 35-day-old animals, thus demonstrating the identity of the active site of the enzyme from old and young populations. The second line of evidence which leads us to conclude that the active enzyme detected in older populations is the same as the enzyme of younger animals comes from studies of the heat inactivation of the enzyme. In Fig. 5 the effect of exposure of crude enzyme preparations of animals 5 and 45 days old to elevated temperature is shown. The inactivation curves are virtually parallel, thus attesting to the identity of the two active enzyme populations. These results strongly indicate that in old animals the population of enzyme molecules consists of a proportion of fully active molecules and a large amount of altered inactive molecules.

The alteration in enzyme structure resulting in inactive molecules may arise from one of three possible mechanisms or any combination of them: (a) modification of a large proportion of enzyme molecules in the old animal after their synthesis; (b) changes in primary structure caused by errors during the synthesis of enzyme molecules; and (c) progressive accumulation of an inhibitor of enzyme function. Our experiments testing enzyme activity in mixed preparations from young and old animals eliminate the third possibility. The first possibility seems most likely, for random errors in protein synthesis would result in a proportion of partially
active enzyme molecules; our results demonstrate that the enzyme population is composed of either inactive or fully active molecules for isocitrate lyase. The nature of the chemical modification of the isocitrate lyase molecule which lead to their inactivation is unknown.

It is interesting to note that Palmer and Papacostantinou have recently reported a post-synthetic chemical modification in a subunit of adult α-crystallin, although this modification does not result in immunological change of the resultant subunit.

Our findings also indicate that investigations of total enzyme activity as a function of age cannot be expressed merely in terms of specific activities. If for a given unit of enzyme activity the old cell synthesizes a considerably larger number of molecules than the young cell, the burden on the aged cellular system in terms of energy expenditures and synthetic capacity is much greater and probably has deleterious consequences. At present, it is not known, however, at what levels of faulty proteins the physiological functions of eukaryotic cells become altered.

The same approach must be repeated with purified enzyme from other cellular systems and other organisms in order to investigate the universality of the phenomenon reported here. If found to be universal, it should have important implications for the understanding of the mechanisms of ageing.

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X-linked Gene for Testicular Feminization in the Mouse

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The condition of testicular feminization in mice resembles that in man and other mammals. It is clearly X-linked. There is no evidence at present that the gene for testicular feminization is involved in mouse spermatogenesis, at least up to the spermatocyte stage.

X-linked Inheritance of Testicular Feminization in Mice

A female mouse heterozygous for the X-linked genes tabby (Tα) and blotchy (Blο) of genotype Tα Blο+/+, when mated to a normal male, produced ten apparently female progeny which expressed the Tα and Blο phenotypes of hemizygotes or homozygotes. On dissection they proved to have testes, and chromosome counts showed them to have forty chromosomes. It was therefore thought that they must be genetic males with chromosomal constitution XY, and this was further confirmed by the sex ratio among the original female's offspring. Including the ten anomalous animals as females, there were fifty females and twenty-one males, a significant deficiency of normal males. The similarity to testicular feminization in man was immediately obvious, and the gene postulated to be carried by the feminized males was given the name and symbol testicular feminization, Tfσm.

Of the twenty-one normal males produced by the original female carrier, only two were Tα Blο/Y and nineteen were +/+Y, whereas there were ten phenotypic females presumed to be Tα Blο/Y and carrying Tfσm. This suggested that Tfσm was X-linked, and further work was directed to confirming the X-linkage, locating the gene on the X-linkage map, and obtaining Tfσm/Y animals without other mutant genes (such as Tα and Blο) so that the gene effect could be studied in an otherwise normal animal.

Linkage Studies

If the condition was indeed caused by an X-linked gene, as it appeared, then, in the absence of crossing over, those daughters of the original female which were Tα Blο/+ would be expected to carry Tfσm, and those which were +/+ would not. Fourteen such daughters were bred from, three which looked Tα Blο/Y and eleven which looked +/+ . All three Tα Blο/+ produced some apparently female offspring not showing the Tα + phenotype, and which had testes. This last female therefore carried an X-chromosome in which the Tfσm gene had been separated by crossing-over from Tα and Blο.

Data from this female and her descendants (Table 1)