Turnover of Genetically Defined Catalase Isozymes in Maize
(density labeling/synthesis/degradation/starch-gel electrophoresis)

PETER H. QUAIL* AND JOHN G. SCANDALIOS†

MSU/AEC Plant Research Laboratory, Michigan State University, East Lansing, Mich. 48823

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ABSTRACT The turnover of two homotetramers of catalase, V and Z, specified by genes at two separate loci, has been studied during germination of maize seed by the techniques of density labeling and starch-gel electrophoresis. Both isozymes were shown to be turning over during this time. However, Z accumulates because the rate of synthesis exceeds the rate of degradation, whereas V slowly disappears because the rate of degradation exceeds the rate of synthesis. Evidence is also presented that the rate of synthesis of Z exceeds that of V, which suggests that this may be a major factor in the differential expression of the two catalase genes.

Maize catalase (H2O2: H2O oxidoreductase, EC 1.11.1.6) is a tetramer (1). The products of catalase genes at two distinct loci, Ct1 and Ct2, have been detected and shown to be inherited independently of each other (Scandalios, manuscript in preparation). When present together at the same stage of development, the two gene products (subunits) interact to form active tetramers. However, from the time of pollination to just prior to seed maturation, only the product of the Ct1 locus is present in the developing caryopsis of inbred lines homozygous at this locus. Thus, only one isozyme (the homotetramer) is active during this time. The product of the Ct2 locus is first detected in the dry seed and increases dramatically during the first 3 days of germination. The Ct2 gene product, on the other hand, slowly decreases in activity during germination and ultimately disappears. During the time that both classes of subunits are available, five species of active tetramers are detected—two homotetramers and three heterotetramers.

The above observations raise the following questions. Is the newly appearing product of the Ct2 locus synthesized de novo and does it turn over as it accumulates? Does the disappearance of the product of the Ct2 locus represent degradation in the absence of further synthesis or are the subunits turning over as they disappear? Do the rates of synthesis and degradation of the two gene products differ from one another? We have attempted to answer these questions by the techniques of starch gel electrophoresis (2) and density labeling (3, 4).

The inbred line W64A, homozygous for the Ct2 allele at the Ct2 locus, was used in this study. The polypeptide product of this allele is designated subunit V; the homotetramer is V. The polypeptide product of the Ct2 locus is tentatively designated subunit Z; the homotetramer is Z. In this inbred line, the Z isozyme has a greater electrophoretic mobility than the V isozyme.

MATERIALS AND METHODS

Growth and extraction of seedlings

Seeds of maize, inbred line W64A, were germinated between moistened filter papers in Petri dishes in the dark at 24°C. The papers were moistened either with 10 mM K4NO3 in H2O (3N-H2O) or with 10 mM (99 atom %) K214NO3 in 70% D2O (3N-D2O). In the chase experiments, seeds were germinated for 36 hr in 3N-D2O rinsed for 20 min with several changes of water, and then placed in 3N-H2O for the chase period. Twenty scutella from each treatment were homogenized with sand in a mortar and pestle in 1 ml of 25 mM glycylglycine buffer (pH 7.4) and centrifuged at 20,000 × g for 10 min. The crude supernatant was used directly for electrophoresis.

Electrophoresis

Starch-gel electrophoresis and staining for catalase were performed as described (2). For preparative purposes the entire extract from twenty scutella was applied to a single starch gel. After electrophoresis, a thin slice of the gel was stained to facilitate location of the V and Z isozymes. Strips corresponding to these two isozymes were cut from the remainder of the gel and centrifuged at 30,000 rpm for 30 min in an International B60 ultracentrifuge equipped with an SB206 rotor. Each strip yielded 2-2.5 ml of fluid; this was used directly for centrifugation in CsCl. This method resulted in a preparation of Z free of the other four isozymes as judged by electrophoresis. The V eluate was contaminated to some extent by the other four isozymes, because of ‘trailing’. The degree of contamination was difficult to estimate by electrophoresis because tetramers composed predominantly of the V subunits were less stable under the electrophoretic conditions than were tetramers composed predominantly of Z subunits. This considerably exaggerated the proportion of contaminant isozymes upon electrophoresis.

Density gradient centrifugation

The procedure was essentially that of Filner and Varner (4). Each tube contained 1 ml of saturated CsCl, 2 ml of gel eluate, and 2 μg of lactate dehydrogenase (EC 1.1.1.27; Sigma) as a marker, all uniformly mixed. The tubes were centrifuged at 40,000 rpm for 65 hr at 3°C in an International B-60 ultra-
Fig. 1. Zymogram showing the change in catalase isozymes during the first 6 days after seed imbibition. The Vt homotetramer is specified by the Cht allele at the Ch locus. The Zt isozyme is specified by a second locus Cht. Isozymes of intermediate electrophoretic mobility result from the interaction of the two gene products to form heterotetramers. O, point of sample insertion.

centrifuge equipped with an SB405 rotor. The tubes were punctured with a no. 22 needle and three-drop fractions were collected in the cold. The refractive index of every tenth fraction was determined on a Bausch & Lomb Abbe-32 refractometer and converted to density units. LDH activity was determined according to the method of Kornberg (5). Catalase activity was determined with a Clark oxygen electrode by a modification of the procedure of Rorth and Jensen (6). Recovery of catalase activity from the gradient was always greater than 70%, with no quantitative differences between isozymes.

RESULTS

Fig. 1 shows the changes that occur in the catalase isozyme pattern in maize scutella during the first 6 days after seed imbibition. In vitro mixing experiments (Scandalios, manuscript in preparation) have established that the heterotetramers detected are the result of in vivo random association of the subunits and are not an artifact of the extraction procedure. This indicates that the V and Z subunits are both present in the same compartment of the same cell at the same time.

Both catalase isozymes Vt and Zt become density labeled during the first 36 hr of germination (Fig. 2). This indicates that both classes of subunits are synthesized de novo. The use of D2O as one of the density labels raises the possibility that, were catalase a glycoprotein, the density shift could be entirely the result of deuteriation of a carbohydrate moiety without synthesis of the protein moiety (7, 8). However, the low inherent density of the catalase molecules (1.290 g/ml) and the density shift of up to 0.019 g/ml upon labeling are strong evidence that the protein moieties are synthesized de novo. To obtain a density shift of 0.019 g/ml by deuteriation of a postulated carbohydrate moiety without synthesis of the protein moiety, the catalase would need to be at least 45% carbohydrate. A density of 1.290 g/ml for the unlabeled enzyme renders this possibility unlikely. For example, horse radish peroxidase At (9), known to be only 20% carbohydrate (10), has a density of 1.349 g/ml (8). A similar argument indicates that deuteriation of a lipid moiety of catalase does not explain the observed density shift.

Since there is a net loss of Vt during germination (Fig. 1), this isozyme is obviously being degraded as well as synthesized and is therefore turning over as it disappears. However, it cannot be determined from the above data whether or not Zt is also degraded. A pulse-chase density labeling experiment was therefore designed to investigate this possibility and to compare the rates at which the Vt and Zt isozymes decrease in density during the chase period. After a 36-hr labeling period in 3H-N-D2O (designated 'fully labeled'), the subsequent decrease in the buoyant density of each of these isozymes was
followed during a 24-hr chase period in $^{14}$N–$\text{H}_2\text{O}$. Fig. 3 shows the density gradient activity profiles obtained for the unlabeled and fully labeled catalase isozymes, together with those for chase periods of 6, 12, 18, and 24-hr. Both isozymes shift in density back towards the density of the unlabeled protein during the chase period. This is further evidence that $V^4$ is turning over.

However, since there is a net increase in the activity of $Z^4$ during the chase period, the shift in density of the $Z^4$ peak is not in itself sufficient evidence that this isozyme is being degraded. This is because the position of the enzyme profile is determined by the relative proportions of labeled and unlabeled molecules in the population at any one time.

A rapid rate of synthesis during the chase period could elevate the proportion of new, unlabeled molecules to a level several-fold that of the old, labeled molecules; hence, there would be no necessity to postulate the degradation of any labeled molecules. Under these conditions the peak center would also shift with time towards the unlabeled position at a rate dependent entirely on synthesis and not degradation. To establish that the shift in density observed for $Z^4$ is the result of degradation as well as synthesis, it is necessary to consider additional parameters. It is known that (a) there is only a three-fold increase in $Z^4$ activity in the 24 hr of the chase period; (b) this increase is approximately linear with time; and (c) the density of the $Z^4$ peak after a 24-hr chase is 1.298, midway between the fully labeled and unlabeled proteins. With these constraints, it is possible to construct theoretical curves simulating zero-degradation conditions and determine how closely these approximate the observed data.

To achieve the observed density of 1.298 in the absence of degradation, the newly synthesized molecules (67% of the total activity) would have to have a density low enough to compensate for the high density of the undegraded, fully labeled molecules remaining (33%). The theoretical enzyme profiles constructed on this basis were skewed and/or 20–28% broader than the observed $Z^4$ peak. Furthermore, the $Z^4$ peak-width remains constant from 6 to 24 hr of chase time (Table 1). Under zero-degradation conditions, a steadily increasing profile width would be expected as more and more unlabeled molecules were added to the population. Since the $Z^4$ profiles are neither markedly skewed nor as broad as expected for

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**Table 1.** Width at half-height for catalase and LDH enzyme profiles after isopycnic equilibrium centrifugation in CsCl

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<th>Chase time (hr)</th>
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zero degradation, it is clear that the observed decrease in mean density was achieved through the degradation of labeled molecules as well as the synthesis of lighter ones. Z4 is therefore turning over as it accumulates.

The center of each of the approximately Gaussian enzyme profiles in Fig. 3 represents the mean density of all the enzyme molecules in the population at that time. The rate at which the mean density approaches that of the unlabeled enzyme during the chase period is therefore a measure of the changing proportions of labeled and unlabeled molecules in the population. This in turn is a function of the relative rates of synthesis and degradation within the population, provided equilibration of the amino-acid pool with unlabeled amino acids is not rate-limiting. The fractional decrease in the mean density of V4 and Z4 with increasing chase time has therefore been plotted as a percentage of the density difference (Δρ) between the unlabeled and the fully labeled molecules in each case and designated 'percent return' (Fig. 4). For V4, the Δρ between unlabeled and fully labeled molecules is 0.015; for Z4 it is 0.019. Fig. 4a presents these data on a linear scale, Fig. 4b on a log scale. Most of the points in Fig. 4 represent the mean of two experiments, and the standard errors are included. The time required for the mean density to decrease by 50% of the Δρ is 22 hr for Z4 and 47 hr for V4.

DISCUSSION

Both catalase homotetramers V4 and Z4 turn over in the scutella of maize seeds during germination—Z4 as it accumulates; V4 as it disappears. The accumulation of Z4 indicates that synthesis exceeds degradation, whereas the disappearance of V4 indicates that degradation exceeds synthesis. The concentration of each isozyme is, therefore, determined by both the rate of synthesis and the rate of degradation. This fits the general pattern observed for higher organisms (11, 12).

Protein turnover in higher organisms appears to be a general and continuous process and enzyme concentrations may be controlled by independent alterations in either the rate constants of degradation or of synthesis.

Pulse-chase density labeling has been used to measure rates of synthesis and degradation where the density profiles of the labeled and unlabeled enzyme were readily separable into two distinct peaks (13). Initially, we had hoped that quantitative estimates of the rates of synthesis and degradation could be obtained from the kinetics of the density shift (Fig. 4). However, since neither isozyme is in a steady-state condition, it is not possible to estimate from these data the rate constants for synthesis or degradation (12). This is because where enzyme concentrations are changing, the rate of density shift is not only a function of the rate constant of synthesis and the rate constant of degradation but also of the enzyme concentration, which is constantly changing. In addition, it is not known whether or not the rate of equilibration of the labeled amino-acid pool with unlabeled amino acids is rate-limiting to the density shift. This was found to be the case for nitrate reductase in cultured tobacco cells, where considerable recycling of density-labeled amino acids from previously labeled proteins was observed (14).

However, since V4 and Z4 are synthesized simultaneously in the same cell, any differences observed in the relative rates of synthesis or degradation of the two isozymes should be independent of the effects of recycling, provided both are synthesized from the same aminoacid pool. Therefore, although no quantitative estimates of the rates of synthesis and degradation can be made from the data in Fig. 4, some qualitative comparisons should be meaningful.

It is known (Fig. 1) that: (i) the total activity of Z4 > the total activity of V4 at the start of the chase period; (ii) rate of synthesis of Z4 > rate of degradation of Z4; (iii) rate of degradation of V4 > rate of synthesis of V4. Therefore, if the rate of synthesis of V4 were equal to or greater than the rate of synthesis of Z4, the rate of degradation of V4 would automatically be greater than the rate of degradation of Z4. Since total Z4 > total V4 initially, this mandatory higher rate of degradation of V4 would ensure that, if both isozymes were synthesized from the same amino-acid pool, the V4 population would contain, at any point in time, proportionally more of the most recently synthesized, and therefore least dense, molecules than would Z4. Consequently the mean density of

![Graph](https://via.placeholder.com/150)

**Fig. 4.** (a) Time course of the decrease in the densities of catalase isozymes V4 (A—△) and Z4 (O—O) during a 24-hr chase period after 36 hr of labeling. The fractional decrease in density is plotted as a percentage of the density difference (Δρ) between the unlabeled and the fully labeled molecules in each case and is designated 'percent return'. Most points are the means of two experiments. Vertical bars represent standard errors. (b) Semilog plot of data from Fig. 4a.
the V\textsuperscript{4} population would decrease more rapidly than that of the Z\textsuperscript{4} population. Since exactly the opposite relationship is observed (Fig. 4), the rate of synthesis of V\textsuperscript{4} can neither be equal to nor greater than that of Z\textsuperscript{4}. One is left with the conclusion, therefore, that either the rate of synthesis of V\textsuperscript{4} is lower than the rate of synthesis of Z\textsuperscript{4} or the two isozymes are synthesized from separate amino-acid pools that equilibrate at different rates. Since the two gene products are so closely related, are synthesized simultaneously in the same cell, and have the same subcellular distribution (Scandalios, manuscript in preparation), it is reasonable to expect that both are synthesized from a common amino-acid pool.

The corn-scutella catalase system presents an opportunity for the study of differential gene expression in a higher plant, as controlled by differential rates of synthesis and degradation of the gene products. In this system, there are two gene products in the same cell, with presumably the same or similar physiological functions and with apparently the same subcellular distribution, and seemingly at the same stage of development, but differing in their rates of synthesis and/or degradation to the extent that one increases dramatically in activity while the other disappears. The present findings leave many questions unanswered. At what level is the differential rate of synthesis of these two gene products controlled? Is the $C_{2}$ gene switched on during germination and the $C_{1}$ gene switched off, leaving a dwindling supply of relatively stable mRNA to continue the synthesis of V subunits until the $C_{2}$ gene becomes established? Or is the rate of synthesis of the V subunits (sufficient to sustain the required concentrations

of catalase from pollination to maturation) suddenly inadequate in the face of an increased rate of degradation in the germinating seedling, thus necessitating the switching-on of a new gene, with a higher rate of synthesis?

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