Plant Physiol. (1991) 97, 1422–1429
0032-0889/91/97/1422/08/$01.00/0

Received for publication April 3, 1991
Accepted July 16, 1991

Turnover of Catalase Heme and Apoprotein Moieties in Cotyledons of Sunflower Seedlings[1]

Rainer Eising* and Benno Süselbeck

Institut für Botanik der Universität Münster, Schlossgarten 3, 4400 Münster, Federal Republic of Germany (R.E.); and Rechenzentrum der Universität Münster, Einsteinstrasse 60, 4400 Münster, Federal Republic of Germany (B.S.)

ABSTRACT

The turnover of catalase apoprotein and catalase heme was studied in cotyledons of sunflower (*Helianthus annuus* L.) seedlings by density labeling of apoprotein and radioactive labeling of heme moieties. The heavy isotope (50% ^2H_2O) and the radioactive isotope ([^{14}C]5-aminolevulinic acid) were applied either during growth in the dark (day 0–2.5) or in the light (day 2.5 and 5). Following isopycnic centrifugation of catalase purified from cotyledons of 5-day-old seedlings, superimposition curve fitting was used to determine the amounts of radioactive heme moieties in native and density-labeled catalase. Data from these determinations indicated that turnover of catalase heme and apoprotein essentially was coordinate. Only small amounts of heme groups were recycled into newly synthesized apoprotein during growth in the light, and no evidence was found for an exchange of heme groups in apoprotein moieties. It followed from these observations that degradation of catalase apoprotein was slightly faster than that of catalase heme. A degradation constant for catalase apoprotein of 0.263 per day was determined from the data on heme recycling and the degradation constant of catalase heme determined previously to be 0.205 per day (R Eising, B Gerhardt [1987] Plant Physiol 84: 225–232).

In comparison with the increment of our knowledge on the biogenesis of peroxisomal proteins in plants in the last several years, only little progress was made in understanding the turnover (degradation) of proteins from plant peroxisomes. Results obtained from a few studies indicate that the general mechanism is one of individual protein degradation rather than a degradation of the whole organelles (1, 8, 9, 11, 12, 22, 27, 28). The concept of individual protein degradation suggests that (at least) initiating steps of the degradative pathway are localized in the peroxisomes. These steps are not necessarily proteolytic processes, but could be any chemical modification (*e.g.* oxidation, dephosphorylation), or a specific export of proteins destined for degradation in other cell compartments.

For catalase, a tetrameric peroxisomal protein containing four heme groups per molecule, it is conceivable that dissociation of prosthetic groups is a step of its intraperoxisomal degradative pathway. The biosynthetic pathway, on the other hand, comprises the intraperoxisomal incorporation of heme groups into catalase apoprotein (14–17, 24). This concept on heme acquirement and dissociation gives rise to the speculation that turnover rates of catalase heme and apoprotein could be different. An asynchronous turnover could be due to an exchange of heme groups (*i.e.* heme groups lost from apoprotein are replaced by newly synthesized ones), or a recycling of heme groups (*i.e.* heme from degraded apoprotein is reincorporated into newly synthesized apoprotein).

Tait (25) generalized that a recycling of heme groups from degraded hemoproteins did not occur in animal cells. Examples for a heme exchange were reported for Cyts (3, 6, 19), whereas Poole (20) found a coordinate turnover of catalase heme and catalase apoprotein in rat liver.

A general methodological problem of studies on the relationship between apoprotein and heme turnover is a recycling of radioactive amino acids used to label the apoprotein moiety (4, 5). Recycling of amino acids leads to an underestimation of apoprotein turnover. Therefore, a disparity between measured rates of apoprotein and heme turnover could have been due to recycling of labeled amino acids (19–21).

Recently, we reported a quantitative analysis on the turnover of catalase heme in cotyledons of sunflower seedlings (8, 9). The goal of the studies reported in this paper was to find out whether heme and apoprotein of catalase undergo a coordinate (synchronous) turnover and, by quantifying eventual differences, to determine the degradation constant of catalase apoprotein. To avoid methodological problems due to recycling of radioactive amino acids, we used an alternative experimental approach including density labeling of catalase apoprotein and simultaneous radioactive labeling of catalase heme in the cotyledons. Data from isopycnic centrifugations of catalase were evaluated using a numerical mathematical approach and a computerized curve fitting program described recently (10).

MATERIALS AND METHODS

Plant Growth Conditions

Seed coats were removed from dry sunflower achenes (*Helianthus annuus* L. cv Spanners Allzweck, HESPA-Sonnenblumen GmbH, Straubing, FRG) and embryos were soaked for 16 h in water. After removing one of the cotyledons (8), embryos were grown for 2.5 d in the dark at 30°C. Counting of the days of growth started with the transfer of the embryos to 30°C. During the first day of growth, the seedlings were

[1] Financially supported by a grant from the Deutsche Forschungsgemeinschaft (FRG) to R.E.

held on moist filter paper. Thereafter, they were mounted in a rack, and the radicles were placed into water. From day 2.5 of growth on, the seedlings were grown as described (2) in continuous white light at 150 μE m^{-2} s^{-1}. The growth period from day 0 to 2.5 was denoted stage I, that from day 2.5 to 5 stage II.

Labeling of Catalase

For density labeling of catalase in the cotyledons, sunflower seedlings were grown on 50% (v/v) ^2H_2O (diluted with tap water from 99.5% ^2H_2O). Heavy water was applied either during stage I (after soaking embryos in 50% [v/v] ^2H_2O) or stage II of cotyledon growth. The isotope change was always performed at day 2 of growth. Heme groups in catalase were radioactively labeled *in vivo* as described (8) by applying [4-^{14}C]ALA[2] to the cotyledon surface. To study heme recycling, the labeling program comprised a pulse with [^{14}C]ALA (at day 0) followed by a chase with [^{12}C]ALA (at day 1). To determine a heme exchange in catalase, [^{14}C]ALA was applied to the cotyledons during stage II. For use as internal marker, catalase apoprotein was radioactively labeled by applying 0.5 nmol (in 3.5 μL) L-[4,5-^{3}H]leucine (2.1 GBq μmol^{-1}) to the surface of the cotyledons either at day 0 or day 2.5 of growth.

Determination of Light and Heavy Catalase

The amounts of light and heavy catalase in heterogeneous ^{14}C-labeled populations were determined by isopycnic centrifugation on CsCl gradients as described in detail (10). Internal markers (*i.e.* ^{3}H-labeled light or heavy catalase) were used to determine the peak positions of light or heavy catalase directly in the gradients containing the heterogeneous populations of ^{14}C-labeled catalase.

After the labeling programs described above, catalase was purified from peroxisomes of 5-d-old cotyledons (7), mixed with either light or heavy ^{3}H-labeled marker catalase, and centrifuged on CsCl gradients. Gradients were fractionated from the bottom. After discarding the first 0.5 mL, 2-drop fractions (40 μL) of the remaining gradient were collected into scintillation vials. Refractive indices were determined for every ninth drop and converted into density units. Radioactivity (^{14}C and ^{3}H decay rates) in each fraction was determined using the double label mode of a LKB Wallac 1217 Rackbeta counter.

The mathematical operations to determine the amounts of light and heavy ^{14}C-labeled catalase in a heterogeneous population by superimposition curve fitting were detailed recently (10) and are therefore only shortly summarized. Distribution of a homogeneous population of catalase in a CsCl gradient was described by a Gaussian curve (Eq. 1):

$$y = f(x) = A\frac{\sqrt{R}}{\sqrt{2\pi}}e^{-\frac{R}{2}(x-M)^2} \quad (1)$$

where A was the area under the curve (corresponding to the amount of catalase in the gradient), R the reciprocal of the variance, and M the mean value (x axis position of the profile peak). All parameters were determined by a suitable algorithm. The distribution of a mixture of light and heavy catalase was described by a function (Eq. 2) resulting from the arithmetical sum of two Gaussian curves:

$$y = A_1\frac{\sqrt{R_1}}{\sqrt{2\pi}}e^{-\frac{R_1}{2}(x-M_1)^2} + A_2\frac{\sqrt{R_2}}{\sqrt{2\pi}}e^{-\frac{R_2}{2}(x-M_2)^2} \quad (2)$$

The evaluation of A_1 and A_2 (*i.e.* the amounts of heavy and light catalase in the gradient) by an algorithm required experimental determinations of the mean values (M_1, M_2) and the total area ($A_t = A_1 + A_2$) under the superimposition curve. By single Gaussian curve fitting to the distribution of the internal marker, one of the two mean values was always determined directly from the gradient containing the heterogeneous population of ^{14}C-labeled catalase. From this mean value, the second one was calculated using the slope of the density gradient and the density difference of 7.9 g L^{-1} between homogeneous light and heavy catalase (10). Total area A_t under the superimposition curve was determined by single Gaussian curve fitting to the ^{14}C distribution profile. This approach for the determination of A_t proved to be justified (10).

Values for the unknown parameters in Equation 2 were determined by minimizing Equation 3:

$$\sum_{i=1}^{n}(f_1(x_i) + f_2(x_i) - y_i)^2 = \sum_{i=1}^{n}\left(A_1\frac{\sqrt{R_1}}{\sqrt{2\pi}}e^{-\frac{R_1}{2}(x_i-M_1)^2} + A_2\frac{\sqrt{R_2}}{\sqrt{2\pi}}e^{-\frac{R_2}{2}(x_i-M_2)^2} - y_i\right)^2 \quad (3)$$

where x_i and y_i represented the measured values (fraction number and radioactivity in the fractions). Initial estimate for R_1 and R_2 was the value of the parameter R (Eq. 1) from the single Gaussian curve fitting of the internal marker. A_2 was replaced by $A_t - A_1$, and for A_1 the half of A_t was taken as initial estimate. The values determined for M_1, M_2, and A_t were kept fixed.

Statistical Analyses

Statistical significance of differences between means and probability distributions was determined by Student's t test and by the U test of Mann and Whitney (18) with a significance level of P = 0.05.

RESULTS

Experimental Approach

Growth of sunflower cotyledons in the first 5 d after germination was divided into two stages (8). Stage I comprised the period from day 0 to day 2.5 (growth in the dark), stage II the period from day 2.5 to day 5 (growth in continuous light). In the course of stage II, glyoxysomes in the cotyledons were transformed to leaf-type peroxisomes (8). Catalase synthesized during stage I and II was denoted catalase I and

[2] Abbreviation: ALA, 5-aminolevulinic acid.

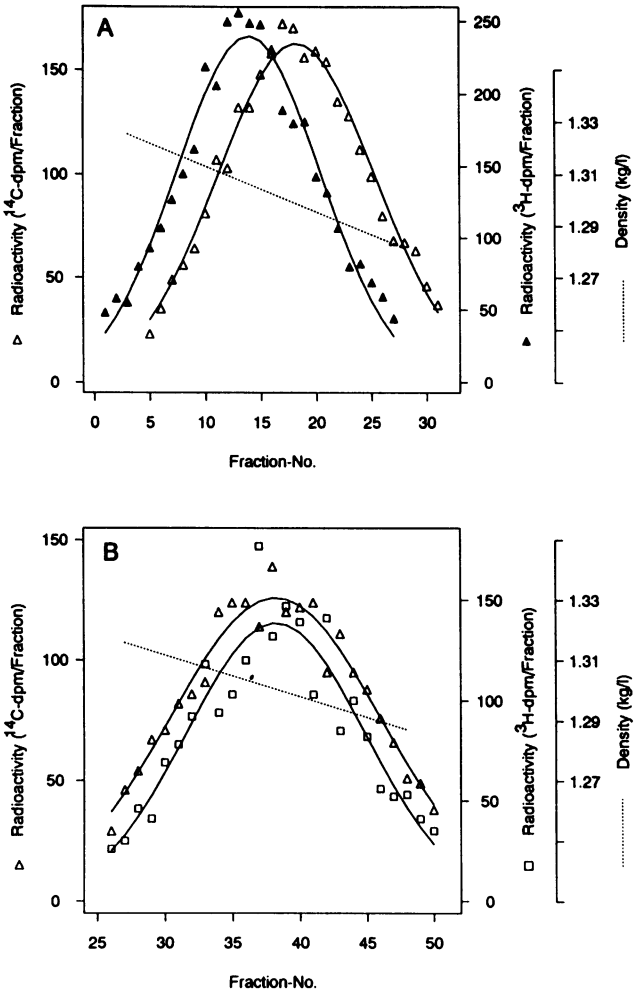

Figure 1. Density of catalase II after an isotope change from ^1H_2O to ^2H_2O (A) or from ^2H_2O to ^1H_2O (B). The isotope change was performed at day 2, and catalase apoprotein II was labeled by application of [^{3}H]leucine to the cotyledons at day 2.5. Catalase purified from 5-d-old cotyledons was centrifuged with ^{14}C-labeled light catalase I. The difference in density between ^{3}H-labeled heavy catalase II and ^{14}C-labeled light catalase I was 7.6 g L^{-1} in experiment A. No difference in density was found between ^{3}H-labeled light catalase II and ^{14}C-labeled light catalase I in experiment B.

catalase II, respectively. Correspondingly, the denotions apoprotein I and apoprotein II, and heme I and heme II, were used to indicate the stage in which the component was synthesized.

The turnover of catalase heme and apoprotein was studied during stage II using a method (10) that involved density labeling (by ^2H_2O) of catalase apoprotein combined with radioactive labeling (by [^{14}C]ALA) of heme groups. Depending on the labeling program for ALA, the method allowed visualizing either an exchange of heme groups by newly synthesized ones (exchange of heme I by heme II in apoprotein I), or a recycling of heme groups into newly synthesized apoprotein (recycling of heme I into apoprotein II). When heme exchange was to be studied, labeling of heme moieties by ALA was performed during stage II. Recycling of heme groups was analyzed after pulse-chase labeling of catalase heme during stage I. The transfer of the seedlings from ^2H_2O to ^1H_2O (or vice versa) was always performed at the end of stage I. This led to a heterogeneous catalase population consisting of heavy catalase I and light catalase II (or vice versa) at the end of stage II. In all cases, catalase was purified from the cotyledons at day 5. Following isopycnic centrifugation on CsCl gradients, the amounts of exchanged or recycled heme were determined from the distribution of radioactivity (^{14}C-labeled heme groups) between light and heavy catalase (catalase I and catalase II). The mathematical approach (10) for these determinations required an experimental determination of the peak positions of light and heavy catalase in the gradients (M_1 and M_2 in Eq. 2). Therefore, the gradients always contained either light or heavy ^{3}H-labeled catalase serving as internal markers.

Certain requirements must be fulfilled if the described method is to be used. In previous reports, it was already shown that purified catalase was free of contaminating radioactivity and that the radioactivity in catalase was confined to heme moieties (7, 8). It was still to be tested whether application of ^2H_2O during stage I led to the same density shift of catalase as an application during stage II, and whether only light catalase was synthesized during stage II after an isotope change from ^2H_2O to ^1H_2O at the end of stage I. In addition, the influence of ^2H_2O on the turnover of catalase had to be investigated.

Density Labeling of Catalase during Stage I and Stage II

For density labeling of catalase I, embryos of sunflower achenes were soaked in 50% (v/v) ^2H_2O and grown on the same solution during stage I. This led to a population of heavy catalase I with a density of 7.9 g L^{-1} (SD = 0.8 g L^{-1}; n = 9), higher than that of light catalase (10). After applying ^2H_2O during stage II and concurrently labeling apoprotein II by

Table I. *Influence of ^2H_2O on Catalase Activity, Content, and Turnover*

Sunflower seedlings were grown on ^1H_2O or ^2H_2O according to three different programs: ^1H_2O in stage I followed by ^2H_2O in stage II (labeling program $^1H_2O/^2H_2O$); ^2H_2O in stage I followed by ^1H_2O in stage II (labeling program $^2H_2O/^1H_2O$); ^1H_2O in both stage I and II (control). The specific activity of catalase (μkat × nmol heme^{-1}), total catalase content of the cotyledons (pmol catalase heme × cotyledon^{-1}), the synthesis rate of catalase heme in stage II (pmol catalase heme × cotyledon^{-1} × 2.5 d^{-1}) and half-life of the heme groups in catalase I during stage II (d) were determined as described previously (8, 9).

Measured Parameter	Time of Growth	Labeling Program	
		$^1H_2O/^2H_2O$	$^2H_2O/^1H_2O$
		% of control	
Specific activity	day 2.5	90	82
	day 5	105	92
Total catalase	day 2.5	90	84
	day 5	83	93
Synthesis rate	stage II	96	126
Half-life	stage II	82	81

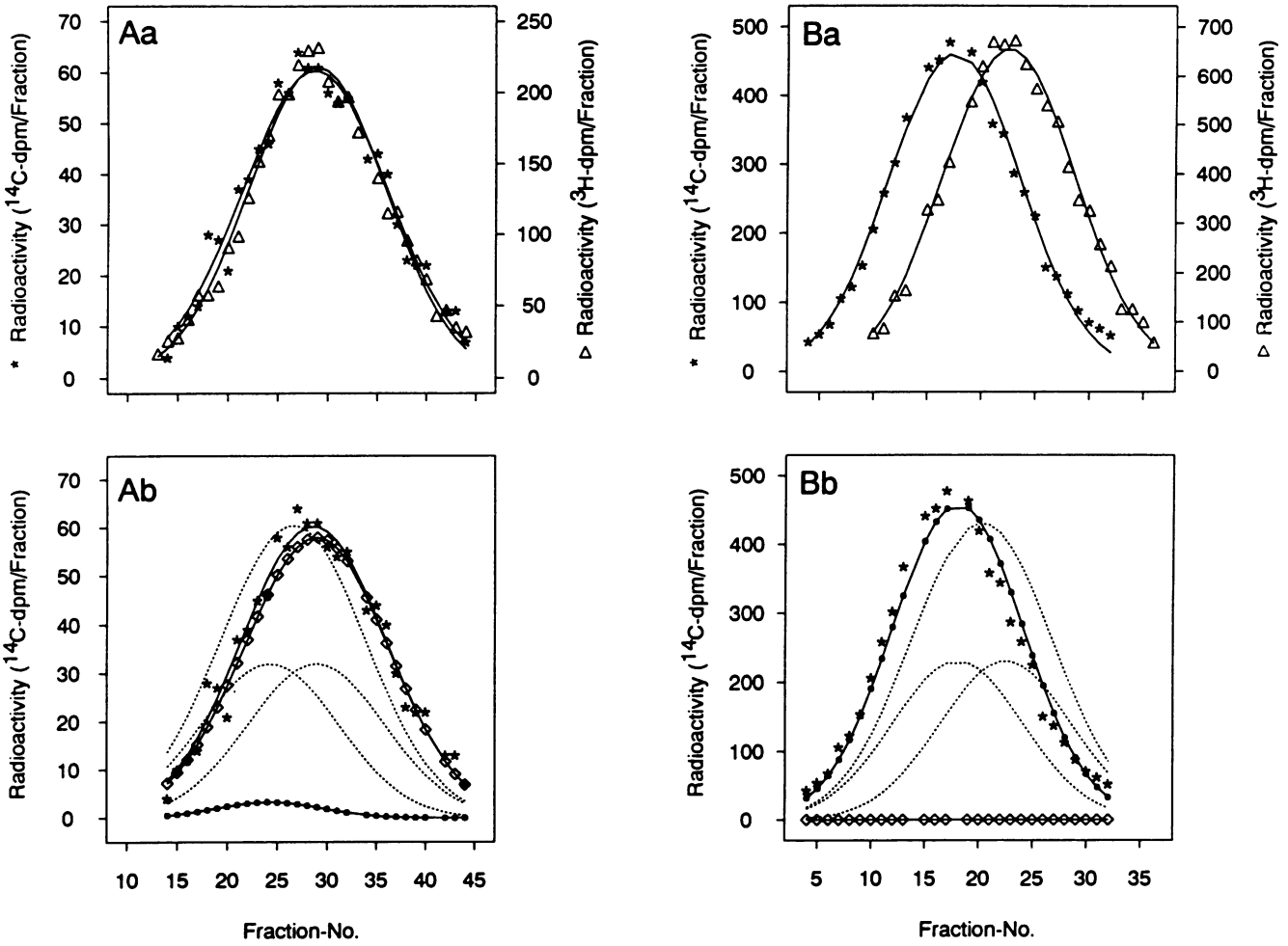

Figure 2. Superimposition curve fitting for the determination of a heme exchange in catalase. (A) Seedlings grown on ^2H_2O during stage I and on ^1H_2O during stage II. (B) Seedlings grown on ^1H_2O during stage I and on ^2H_2O during stage II. In both experiments, [^{14}C]ALA was applied to the cotyledons in stage II. After these labeling programs, heme exchange would lead to ^{14}C label in heavy (A) or light (B) catalase I. At day 5, catalase was purified from the cotyledons and centrifuged together with a [^{3}H]leucine-labeled light marker catalase on CsCl gradients. Decay rates for ^{14}C (*) and ^{3}H (Δ) were determined in the fractions collected from the bottom of the gradients. (a) Single Gaussian curve fitting to the distributions of the [^{14}C]heme-labeled catalase and of the [^{3}H]leucine-labeled light marker catalase. Single Gaussian curve fitting provided values for the total area under the ^{14}C profile and the peak position of light catalase in the gradient. Both values were needed for superimposition curve fitting (10). (b) Superimposition curve fitting to the distribution of ^{14}C-labeled catalase (* = measured values). Dotted lines: initial Gaussian curves for light and heavy catalase, and initial superimposition curve. Solid lines: final Gaussian curves representing calculated amounts of [^{14}C]heme in light (◇) and heavy (●) catalase in each fraction; final superimposition curve (—), which coincides with the Gaussian curve for heavy catalase in (Bb). In experiment A, 4% of total ^{14}C label in catalase was present in heavy catalase I. In experiment B, no ^{14}C label was found in light catalase I. These results indicated that heme exchange was very low or not detectable.

[^{3}H]leucine, the density of ^{3}H-labeled heavy catalase II differed by 7.7 g L^{-1} (SD = 0.7 g L^{-1}; n = 4) from that of light catalase (Fig. 1A). When the ^{3}H-labeled heavy catalase II was centrifuged with ^{14}C-labeled heavy catalase I, the difference in density between ^{3}H- and ^{14}C-labeled catalase was always (n = 4) smaller than 0.8 g L^{-1} (not shown). These results indicated no difference in density between heavy catalase I and heavy catalase II.

When the seedlings were transfered from ^2H_2O to ^1H_2O at the end of stage I, only light catalase was synthesized during stage II. This was indicated by results from experiments in which catalase II was labeled with [^{3}H]leucine after the change from ^2H_2O to ^1H_2O and then centrifuged together with ^{14}C-labeled light catalase (Fig. 1B).

Influence of ^2H_2O on Catalase Turnover and Activity

It was tested to what extent catalase turnover and activity in sunflower cotyledons were influenced by growing seedlings on 50% (v/v) ^2H_2O. The heavy isotope was applied either during stage I or stage II; the isotope change ($^2H_2O/^1H_2O$ or $^1H_2O/^2H_2O$) was always performed at day 2. Control seedlings were grown on ^1H_2O for the whole growth period. The results (Table I) indicated that ^2H_2O did not seriously affect the

Table II. *Quantification of Heme Exchange and Heme Recycling in Catalase*

Heme exchange and heme recycling were determined by superimposition curve fitting after isopycnic centrifugation of purified catalase as illustrated in Figures 2 and 3. The mean value for heme exchange indicates the percentages of ^{14}C-labeled heme II present in apoprotein I (labeling of heme groups during stage II). The mean value for heme recycling indicates the percentages of ^{14}C-labeled heme I present in apoprotein II (labeling of heme groups by pulse-chase in stage I). Control values were obtained by centrifuging homogeneous populations of either light or heavy ^{14}C-labeled catalase and applying the superimposition curve fitting program to the ^{14}C-profile. The mean value for the control indicates the percentage of ^{14}C label obtained for the catalase type not present in the homogeneous populations. Values for exchanged or recycled heme were compared with the control using both Student's *t* test and (in parentheses) the U test of Mann and Whitney (18) with P = 0.05.

Kind of Experiment	Mean	*n*	SD	Test Statistic	P Value	Significance with P = 0.05
	%		%			
Exchange	7.4	13	7.5	−1.40	0.1372	no
				(27.0)	(0.2178)	(no)
Recycling	16.5	8	13.0	−2.59	0.0226	yes
				(9.5)	(0.0307)	(yes)
Control	3.0	7	4.6			

turnover of catalase. Synthesis of catalase heme during stage II was not reduced (maybe even slightly stimulated when the seedlings were transferred from ^2H_2O to ^1H_2O). Half-life of heme groups in catalase I was shortened by less than 20%, indicating a weak acceleration of catalase degradation caused by ^2H_2O. It was concluded from these results that both density labeling programs led to changes that could be tolerated for the studies on the comparison of catalase heme and apoprotein turnover.

Exchange of Heme Groups

To investigate whether heme groups of catalase were exchanged by newly synthesized ones, heme moieties were labeled by applying [^{14}C]ALA to the cotyledons in stage II. Density labeling of catalase apoprotein was performed during stage I. This standard labeling program led to nonradioactive heavy catalase I and light catalase II containing ^{14}C-labeled heme groups in 5-d-old cotyledons. If an exchange of heme groups occurred during stage II, a ^{14}C-labeled heavy catalase was to be expected (incorporation of ^{14}C-labeled heme II into heavy apoprotein I). Therefore, it was necessary to analyze, by isopycnic centrifugation, whether the heavy fraction of catalase from 5-d-old cotyledons contained ^{14}C label.

As a control of the isotopic effects of ^2H_2O, the standard labeling program for the heavy isotope was inverted in some experiments, *i.e.* seedlings were grown on ^1H_2O during stage I and on ^2H_2O during stage II. After this alternative labeling program, a ^{14}C-labeled light catalase was to be expected if heme groups in light catalase I were exchanged by ^{14}C-labeled heme II.

After the described labeling programs, the amounts of ^{14}C label in light and heavy catalase were determined by isopycnic centrifugation and superimposition curve fitting. Figure 2 illustrates the curve fitting procedure and the results from two experiments conducted with both the standard ($^2H_2O/^1H_2O$) and the alternative ($^1H_2O/^2H_2O$) density labeling program. Only low, if any, ^{14}C label was found in apoprotein I independent of the labeling program for heavy water. Apoprotein I contained an average of 7.4% of total ^{14}C-labeled heme groups incorporated into catalase during stage II (Table II). So, a heme exchange in apoprotein I quantitatively was of almost no importance when compared with the incorporation of heme groups into apoprotein II.

Control experiments were performed to evaluate whether the result positively demonstrated the process of a heme exchange in catalase. Homogeneous catalase, either light or heavy, was subjected to the complete procedure of centrifugation and superimposition curve fitting. The catalase type not present in the gradients was evaluated to represent 3% of the total (Table II). This value was the background of the method due to errors in measurements. The difference between the background and the value for the amounts of heme II in apoprotein I was not statistically significant at the 5% level (Table II). Therefore, we came to the conclusion that there was no positive evidence for an exchange of heme groups in catalase apoprotein I.

Recycling of Heme Groups

The standard labeling program for studies on a recycling of heme groups comprised pulse-chase labeling of heme moieties by ALA and concurrent density labeling of apoprotein during stage I, followed by growth of the seedlings on ^1H_2O during stage II. Consequently, total catalase in 5-d-old cotyledons consisted of ^{14}C-labeled heavy catalase I and nonradioactive light catalase II. Recycling of heme I into apoprotein II would result in a ^{14}C-labeled light catalase. As in the studies on a heme exchange, application of ^2H_2O was performed in some experiments during stage II as a control of isotopic effects of ^2H_2O.

Figure 3 shows the superimposition curve fitting procedure by which the amounts of ^{14}C-labeled heme groups in light and heavy catalase were determined after both the standard ($^2H_2O/^1H_2O$) or alternative ($^1H_2O/^2H_2O$) labeling program. Independent of the labeling program, ^{14}C-labeled heme I was

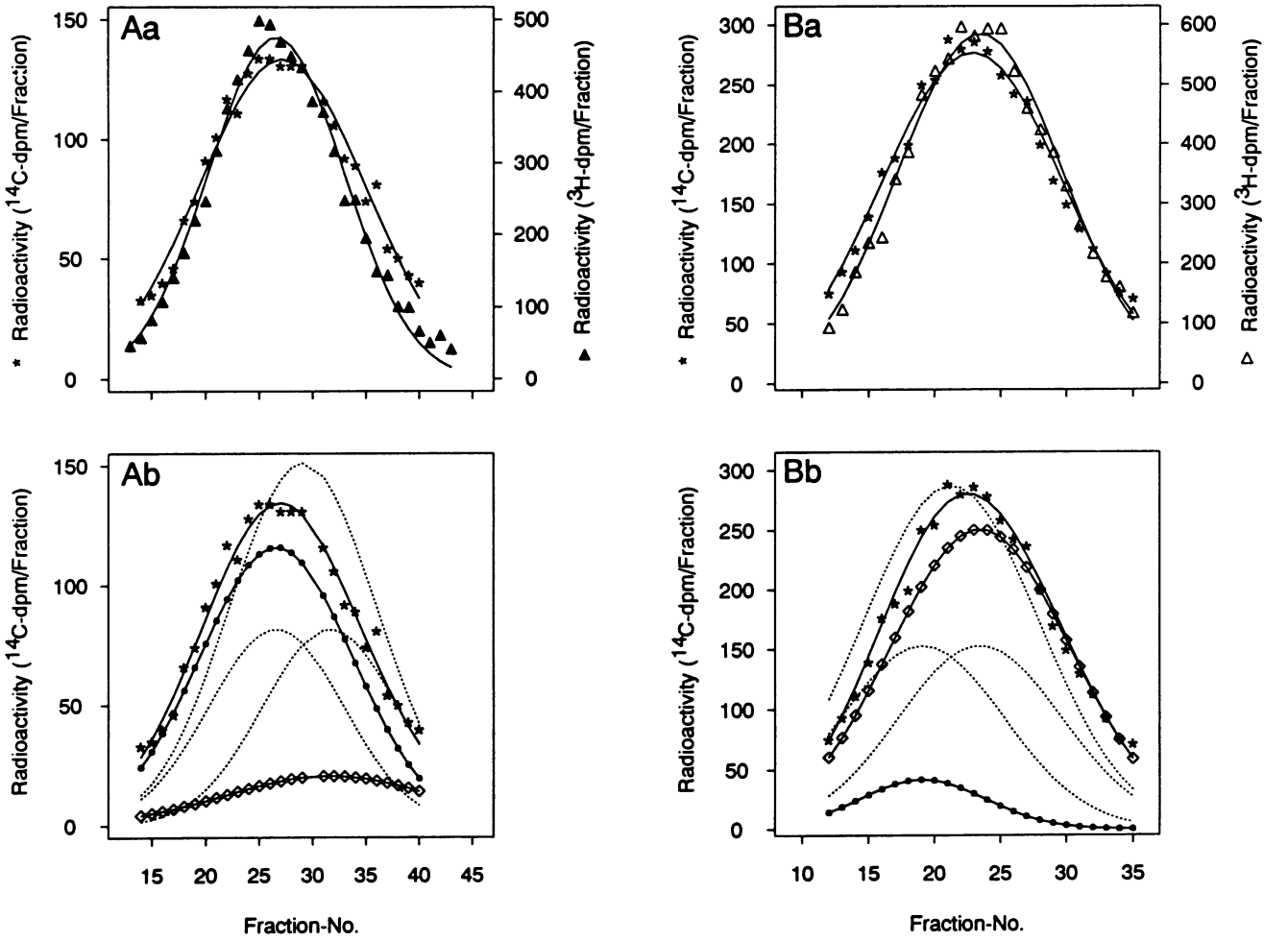

Figure 3. Superimposition curve fitting for the determination of a heme recycling into catalase. (A) Seedlings grown on $^{2}H_2O$ during stage I and on $^{1}H_2O$ during stage II. (B) Seedlings grown on $^{1}H_2O$ during stage I and on $^{2}H_2O$ during stage II. In both experiments, [^{14}C]ALA was applied to the cotyledons at day 0 of growth followed by a chase with [^{12}C]ALA at day 1. After these labeling programs, heme recycling would lead to ^{14}C label in light (A) or heavy (B) catalase II. At day 5, catalase was purified from the cotyledons and centrifuged together with [^{3}H]leucine-labeled marker catalase. Markers were heavy catalase in (A) and light catalase in (B). Decay rates for ^{14}C (*) and ^{3}H (▲ = heavy marker, △ = light marker) were determined in the fractions collected from the bottom of the gradients. For further explanations of graphs and symbols, see Figure 2. In experiment A, 14.2% of total [^{14}C]heme label in catalase was present in light catalase II. In experiment B, heavy catalase II contained 12.5% of total catalase heme label. These results indicated that heme recycling occurred to a low extent.

found in apoprotein II. The average for the amounts of ^{14}C label in apoprotein II was 16.5% of total ^{14}C label in catalase (Table II). This value was significantly ($P = 0.05$) different from the background of the superimposition curve fitting procedure (Table II). Taking the background of 3% into account, it followed that 13.5% of ^{14}C-labeled heme groups in catalase were present in apoprotein II.

In addition to heme recycling, an incorporation of ^{14}C-labeled heme groups into apoprotein II could also have been due to an insufficient chase of [^{14}C]ALA by [^{12}C]ALA leading to the synthesis of ^{14}C-labeled heme II. However, the chase performed in our experiments led to a 70-fold decrease of the specific radioactivity of [^{14}C]ALA in the cotyledons and reached the cellular ALA pool used for synthesis of catalase heme (8, 10). Therefore, the results on the presence of ^{14}C-labeled heme in apoprotein II were considered as evidence for a recycling of heme I into catalase apoprotein II.

DISCUSSION

In sunflower cotyledons, catalase heme and catalase apoprotein exhibited an essentially coordinate turnover during stage II of cotyledon growth. We found no experimental evidence for a heme exchange in catalase apoprotein (replacement of heme I by heme II in apoprotein I), and only small amounts of heme groups were recycled into newly synthesized apoprotein (reincorporation of heme I into apoprotein II). A degradation constant for catalase apoprotein (denoted p) was calculated from the data on heme recycling (this paper) and the amounts of catalase heme I in the cotyledons determined

previously (Fig. 4 in ref. 9). Calculations were done by solving Equation 4 for p with $N_{2.5}$ = 290 pmol catalase heme I × cotyledon^{-1} at day 2.5 and N_5 = 150 pmol catalase heme I × cotyledon^{-1} at day 5. The value for N_5 was obtained by correcting the value of 174 pmol heme I × cotyledon^{-1} at day 5 (Fig. 4 in ref. 9) for 13.5% heme recycling.

$$N_5 = N_{2.5}e^{-pt} \quad (4)$$

The degradation constant p for catalase apoprotein I amounted to 0.263 d^{-1} and was slightly higher than that for catalase heme I, determined to be 0.205 d^{-1} (8). The corresponding half-lives were 2.64 d for apoprotein I and 3.38 d for heme I. This result confirmed our previous interpretations (8) that an observed retardation of catalase degradation during stage II, determined by measuring loss of radioactive heme groups, essentially was not due to a heme recycling. In these preceding studies (8), we used a much less sensitive density labeling method, by which we did not detect a heme recycling. At that time, we came to the conclusion, by rough estimations, that the amounts of recycled heme groups did not exceed 10% of heme I still present at the end of stage II. This upper limit was estimated slightly too low when compared with the results on heme recycling we obtained using the computerized superimposition curve fitting program.

A possible restriction of the conclusions on heme exchange and recycling has to be considered. Usually, protein precursors have by far lower cellular concentrations than the mature proteins. Assuming the unlikely situation that there were high concentrations of catalase apoprotein in precursor pools (relative to apoprotein in mature catalase) at the end of stage I, an incorporation of heme II into apoprotein I was possible without a heme exchange taking place. On the other hand, assuming high concentrations of free heme in the precursor pool (relative to catalase-bound heme) at the end of stage I, heme I could be found in apoprotein II without a heme recycling occurring. However, high turnover times in the precursor pools (relative to the turnover of mature catalase) would largely reduce both events. Lazarow and de Duve (15, 16) reported that pools of catalase precursors (apoprotein and heme) in liver were very small relative to mature catalase. The precursors exhibited half-lives (0.3–2.8 h) much shorter than that of mature catalase (2.2 d). This half-life of liver catalase was very similar to that of catalase apoprotein I in sunflower cotyledons (2.64 d; see above). The concept of small precursor pools subjected to high turnover was confirmed for plant catalase by the results of Kindl (13, 14). The considerations on precursor turnover and pool sizes strongly suggested that our studies were not substantially influenced by large precursor pools exhibiting low turnover.

Another possible explanation for the presence of heme I in apoprotein II is that radioactive heme I came from a storage (intermediate) heme pool the specific radioactivity of which was kept at a high level during the chase. Although this possibility cannot be ruled out unequivocally, it is very unlikely, because we performed a very effective chase for the heme precursor [^{14}C]ALA (see "Results"), and, in addition, endogeneous ALA production increased considerably in sunflower cotyledons between day 2 and 5 of growth (23). Under these conditions, maintaining a heme storage pool of high specific radioactivity would require that this intermediate pool was separated from newly synthesized heme during the chase period. In addition, one had to postulate that, after the chase period, the storage pool was connected to the heme pool in peroxisomes and that no interchange with other cellular heme pools occurred during the transfer of radioactive heme from the storage pool to the peroxisomes.

To further verify the view that heme recycling was the mechanism by which small amounts of heme I were incorporated into apoprotein II, we compared the concentration of catalase-bound heme in peroxisomes to the concentrations of free heme in plastids reported by Thomas and Weinstein (26). They determined a concentration of about 40 pmol free heme/mg plastid protein. The concentration of catalase-bound heme in peroxisomes of sunflower cotyledons was in the range of 2 nmol × mg peroxisomal protein^{-1}, determined from the specific heme content of catalase (7) and the amounts of peroxisomal protein and catalase protein in the cotyledons (9). Because peroxisomes acquire their heme moieties from heme-synthesizing organells (chloroplasts or mitochondria) that have a higher demand for heme than peroxisomes, the concentration of free heme very likely was not higher in peroxisomes than in chloroplasts. It followed that the peroxisomal concentration of catalase-bound heme exceeded that of free heme about 50-fold (or more). These considerations strongly suggested that the presence of heme I in apoprotein II was not due to high amounts of free heme I in peroxisomes during stage II, and consequently supported the conception that heme I was incorporated into apoprotein II by recycling.

It should be stressed that the mechanism by which small amounts of heme I were incorporated into apoprotein II did not have any influence on the calculation of the degradation constant for catalase apoprotein I (Eq. 4). For these calculations, it was not necessary to know the provenance but only the amounts of radioactive heme I in catalase apoprotein II. Because the degradation constant determined for catalase apoprotein I was not distorted by any kind of radioactive label incorporation following the chase, it correctly described the velocity by which catalase was degraded during the transition of glyoxysomes to leaf-type peroxisomes in greening sunflower cotyledons. The result that the "true" degradation constant for catalase apoprotein I was only slightly higher than that determined for catalase heme I confirmed our previous studies on catalase turnover in greening sunflower cotyledons (8, 9). In particular, it verified the conclusion that peroxisome transition was accompanied by a deceleration of catalase degradation. Because synthesis rates of catalase were low during peroxisome transition (9), a delay in degradation was the key factor for maintaining high levels of catalase in peroxisomes changing from glyoxysomal to leaf-type peroxisomal function.

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