Age-Related Changes in the Properties of Enolase from *Turbatrix aceti*[+]

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ABSTRACT: Pure enolase from old *Turbatrix aceti,* a free-living nematode, shows, in addition to a lowered specific activity, several characteristics which differ from those of the enzyme purified from young organisms. "Old" enolase has a greater specific absorbance at 280 nm. An extra tryptophan residue is detectable by titration with *N*-bromosuccinimide, though "young" and "old" enolase show the same total number of residues in the presence of urea. Similarly, two extra tyrosyl groups are titratable in the "old" enolase, although the total number of tyrosine residues is equal in both "young" and "old" preparations. Furthermore, circular dichroism spectra show differences in secondary structure and in the environment of the aromatic amino acids. These differences disappear when the respective proteins are unfolded in 6 M guanidine hydrochloride. The results indicate that a conformational change in the enzyme has taken place in the enzyme isolated from old organisms. The change is not a result of oxidation or reduction of -SH groups as these are equal in number and they all exist in reduced form. Moreover, the altered properties do not appear to involve deamidation, phosphorylation, or partial proteolysis. "Old" enolase is relatively unstable to chromatography on columns of DE-52. During this treatment, the enzyme is partly converted to a denatured form (inactive enolase) which is present in homogenates of old *T. aceti.* The material cross reacts with the antiserum prepared to pure "young" or "old" enzyme. "Young" enolase, though more stable than "old" enolase, can be converted to a product similar to the latter by repeated passage through DE-52. Though not conclusive, the results strongly support the idea that, during aging, "young" enzyme may be converted to "old" enzyme without changes in sequence or involvement of extrinsic factors.

A number of enzymes have recently been shown to become altered in old animals. These enzymes include aldolase from mouse liver (Gershon & Gershon, 1973), superoxide dismutase from rat liver (Reiss & Gershon, 1976), and isocitrate lyase (Reiss & Rothstein, 1975), phosphoglycerate kinase (Gupta & Rothstein, 1976a), aldolase (Reznick & Gershon, 1977), and enolase (Sharma et al., 1976) from the free-living nematode, *Turbatrix aceti.* The common result of the alteration is a reduced specific activity of the enzyme based upon activity/unit of antiserum or activity/mg of pure enzyme. Loss of catalytic ability generally ranges from 40 to 60% in "old" compared with "young" enzymes.[1] Several of these altered enzymes have been purified to homogeneity and characterized in some detail. On the other hand, there are reports that rat liver aldolase (Weber et al., 1976), human muscle creatine kinase and aldolase (Steinhagen-Thiessen & Hilz, 1976), and nematode triosephosphate isomerase (Gupta & Rothstein, 1976b) are unchanged in old organisms.

Besides a loss of specific activity, the "old" enzymes mentioned above seem to show few differences from "young" enzymes. "Old" isocitrate lyase and enolase from nematodes and superoxide dismutase from rat liver show biphasic heat inactivation patterns though "old" mouse liver aldolase (Gershon & Gershon, 1973) and nematode phosphoglycerate kinase (Gupta & Rothstein, 1976a) are unchanged in this respect. No significant change in K_m, molecular weight, charge, or behavior during purification has been observed.

The alteration of old enzymes must be due to either a sequence change or to a postsynthetic modification. Both views have their proponents. Rothstein has recently reviewed the field (Rothstein, 1975, 1977) and has proposed a theory based upon the idea that conformational changes are responsible for the observed alterations in properties of "old" enzymes.

Previous studies of enolase from young and old *T. aceti* (Sharma et al., 1976) suggested that this enzyme by reason of size, stability, and ease of purification, would make an excellent model for more detailed investigation into the properties of altered enzymes. In this paper, data derived from studies of this enzyme demonstrate that the conformation of "old" enolase differs from that of the "young" enzyme. In addition, results are presented which show that, in vitro, "young" enolase can be converted to a product with properties similar to those of "old" enolase. In brief, the evidence available suggests that the changes in "old" enzymes result from altered conformation without a change of sequence.

Materials and Methods

Enzyme Purification and Assays. Procedures were carried out as reported previously (Sharma et al., 1976). Toluenesulfonyl fluoride was added to certain preparations from both young and old organisms and the results compared with preparations made without the protease inhibitor. Immunodiffusion procedures were carried out as previously reported (Sharma et al., 1976).

Isoelectric Focusing of Enolase from T. aceti. Isoelectric focusing of the enzyme was carried out in a column (LKB 8101) containing 110 mL of 1% ampholine (pH 4–6) for 48 h at 400 V at 4 °C. A total activity of 630 units of enzyme was loaded onto the column. After isoelectric focusing, fractions of 2 mL were collected and the pH of each fraction was determined at 4 °C.

Isoelectric focusing on polyacrylamide gel was carried out according to the procedure of Wrigley (1971) in carrier am-

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[1] "Young" and "old" enzymes refer to enzymes obtained from young and old organisms, respectively.

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pholyte of pH 4–6. Approximately 50 μg of protein was loaded for each run.

Heat Inactivation Studies. Enolase (140 μg) in 150 μL of 0.05 M Tris-HCl buffer, pH 7.6, containing 0.001 M $MgSO_4$ was dialyzed overnight against the same buffer. The preparation was heated in a water bath at 52.5 °C in 0.001 M $MgSO_4$. Aliquots of 1–2 μL were assayed for enolase activity at different time intervals.

Repeated Chromatography on Columns of DE-52. Pure samples of "young" and "old" enolase, respectively, were passed through a column of DE-52 (2.5 × 15 cm) using a 0–0.2 M NaCl linear gradient (Sharma et al., 1976). The fractions containing enzyme activity were pooled, concentrated on a PM-10 membrane, and dialyzed against Tris buffer (0.05 M, pH 7.6) for 4 h. Total activity and specific activity were determined. The enzyme samples were then passed again through the column. The DE-52 was prepared in either of two ways:

A. The DE-52 was soaked in 300–400 mL of the Tris buffer for 30–40 min and filtered onto a Buchner funnel. The process was repeated four times. The washed DE-52 was then packed into the column and washed for 3–4 h with approximately 200 mL of Tris buffer.

B. The same procedure was used except that the enzyme was loaded onto the column only after the pH and conductivity of the washes reached the same value as that of the Tris buffer.

Amino Acid Analysis. Samples containing 2 mg of "young" or "old" enolase were dialyzed three times against 4 L of water each time for a total of 72 h. Triplicate samples of the enzymes were hydrolyzed in 6 N HCl and 0.25% phenol in sealed-evacuated tubes for 24, 48, and 72 h, respectively. After the removal of hydrochloric acid, the amino acid content of the hydrolysate was determined on a Beckman 120-C amino acid analyzer. The values for the amino acid composition were averaged except for serine and threonine which were extrapolated to the zero time of hydrolysis and leucine, isoleucine, and valine which were taken from the 72-h hydrolysis run. Half-cystine was determined as cysteic acid after peroxidation of the protein (Hirs, 1967a).

Spectra of "Young" and "Old" Enolase. Complete UV spectra of desalted enzyme samples (230–350 nm) were obtained using a Cary 15 spectrophotometer. Concentrations of enzyme were usually 0.6–0.7 mg of protein/mL.

Trytophan Determination. Pure enolase was first desalted by chromatography on a Sephadex G-25 column (2.5 × 25 cm) equilibrated with water. The enzyme was taken up in 0.05 M acetate buffer, pH 4.0. Tryptophan was then determined by the procedure of Spande & Witkop (1967). Estimations of tryptophan were also performed in the presence of various concentrations of urea. Total tryptophan residues were determined in 8 M urea.

Spectrophotometric Titration. Spectrophotometric titrations were performed on a Cary 15 spectrophotometer using 1.0-cm light path cells over the absorbance range of 0–1.0 at 23 °C using the experimental conditions as described by Donovan (1973). The protein was dialyzed overnight against 0.2 M KCl (250 mL, changed two times) at room temperature and the precipitate was removed by centrifugation. To 1.0 mL of this solution (absorbance 0.5–0.6 at 280 nm) were added different amounts of 0.05–5.0 M KOH such that the maximum amount of alkali added did not exceed 25 μL. The spectra were scanned in the range 240–400 nm against the corresponding protein solution in the same amount of water at 3-, 10-, 30-, and 60-min intervals. The pH measurement was performed using a Beckman Zeromatic pH meter SS-3. Enolase activity was determined by taking 1–2-μL aliquots of the sample 3 min after addition of alkali. The protein concentration selected was such that no denaturation was observed at the higher pH values. The absence of precipitation was also confirmed by comparing the spectra before and after centrifugation of the samples.

Tyrosyl residues were determined by using the maximum extinction coefficient value of 2350 (Sage & Singer, 1962) for phenolate ion of tyrosine.

Protein Determinations. Since enolase from old and young *T. aceti* shows different absorbance at 280 nm, the same molar extinction value for both preparations could not be used and the protein determinations were carried out by the ninhydrin method after the alkaline hydrolysis of the samples (Hirs, 1967b) using bovine serum albumin as the standard. For the spectral studies, duplicate samples of desalted enzyme (Sephadex G-25) were dried in vacuo over P_2O_5 and weighed using a Cahn microbalance. The same volume of eluate coming before the void volume from the G-25 column was taken as a control in these studies. Protein determinations carried out by the two aforementioned methods agreed very well (5% range). Other analyses were carried out by the method of Lowry et al. (1951).

Determination of Sulfhydryl Groups. The -SH titrations were carried out in duplicate essentially according to the procedure of Sedlak & Lindsay (1968) and Habeeb (1972). Pure "young" and "old" enolase, respectively, were dialyzed extensively against 0.2 M Tris buffer, pH 8.2, and 0.02 M EDTA. All other solutions were also made in the same buffer. A suitable amount of protein in 1.0 mL of reaction mixture was mixed thoroughly with 10 μL of 0.01 M 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2)[2] solution and the aborbance at 412 nm was read immediately against a blank without enzyme. The time course of the reaction was then followed. An additional aliquot of Nbs_2 was added when no further color was developing. This addition had no effect, and 60 min later NaDodSO_4 was added to the samples (0.5% final concentration) and the total -SH was determined. After this determination was made, more Nbs_2 was added to assure that the reaction was complete.

Other Analyses. Five batches, respectively, of "young" and "old" enzyme were dialyzed for 72 h against 4 L of water (three changes) and lyophilized. The preparations were used in the following analyses.

N-Terminal Amino Acid. Pure enolase from young and old *T. aceti* was treated with dansyl chloride by the procedure of Gros & Labouesse (1969). The identification procedure was carried out on polyamide plates (Weiner et al., 1972). Ribonuclease was used as a standard for the procedure.

Carboxy-Terminal Amino Acid. Carboxy-terminal analysis of enolase was done according to the method of Ambler (1967). About 3.0 mg of protein was oxidized by the performic acid procedure of Hirs (1967a) and incubated at 37 °C for 3, 6, and 24 h with 15 μg of carboxypeptidase A in 0.3 mL of *N*-ethylmorpholine acetate buffer (0.2 M, pH 8.6), containing 3 M urea. The reaction was stopped by adding 500 μL of 10% Cl_3CCOOH and the mixture was centrifuged. Cl_3CCOOH was removed from the supernatant by five extractions with 2 mL of water-saturated ethyl ether and the aqueous phase was lyophilized. Amino acids released by carboxypeptidase A digestion were identified by the dansylation procedure as described by Airhart et al. (1973).

Circular Dichroism Measurements. Circular dichroism spectra of enolase were recorded at room temperature with a

[2] Abbreviations used: Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); NaDodSO_4, sodium dodecyl sulfate; Gdn·HCl, guanidine hydrochloride.

TABLE I: Amino Acid Composition of "Young" and "Old" Enolase from *T. aceti*.

	no. of residues per mol of enolase	
	young	old
Lys	60	63
His	14	14
Arg	29	27
Asp	104	100
Thr	50	50
Ser	47	47
Glu	74	74
Pro	30	27
Gly	83	91
Ala	112	109
½-cystine[a]	4	4
Val	52	49
Met	10	10
Ile	51	51
Leu	75	74
Tyr	20 (16.9)[b]	21 (16.5)[b]
Phe	24	25
Trp[c]	7	7

[a] Determined as cysteic acid following performic acid oxidation (14). [b] Figures in parentheses give results obtained by titration in 6 M guanidine. [c] Determined by *N*-bromosuccinimide titrations (13).

JASCO spectropolarimeter, Model J-41C. All results are reported as mean residue ellipticity in deg cm^2/dmol MRW of 98. CD measurements were performed in phosphate buffer (0.05 M, pH 7.6). In various experiments, protein samples of 0.6–2 mg/mL (Lowry procedure, Lowry et al., 1951) were dialyzed overnight against the buffer at room temperature and centrifuged. For spectra in the presence of Gdn·HCl the protein was first dialyzed against deionized water for 48 h, lyophilized, and taken up in phosphate buffer containing 6 M Gdn·HCl at a protein concentration of 3–4 mg/mL (weight basis). In the near-UV region (320–255 nm), spectra were recorded in a cell of 1.0-cm pathlength. In the UV region (250–200 nm), the protein was diluted tenfold and a cell of 0.1-cm pathlength was used. Data accumulation was carried out with a processor resolution of 0.1 nm/step, wavelength expansion 5, spectral band width 0.5 nm and number of accumulation, 16. Since the absorbance of Gdn·HCl interferes with CD measurements in the far-UV region, spectra of the enzyme in Gdn·HCl were recorded only in 320–250-nm region.

Trypsin Inactivation of "Young" and "Old" Enolase. To 13 μg of "young" and "old" enolase, respectively, in 50 μL of 0.05 M Tris-HCl buffer (pH 7.6) containing 0.001 M $MgSO_4$, was added 10 μL of the same buffer containing 4 μg of trypsin (specific activity, 278 units/mg). The solution was incubated at 38.5 ± 0.50 °C and samples were removed periodically and assayed immediately for enolase activity.

Results

The purification procedure, as previously reported (Sharma et al., 1976), yielded pure "young" and "old" enolase based on electrophoretic and immunological criteria. In addition, isoelectric focusing showed absolutely no difference between "young" and "old" preparations. Each protein focused as a single, sharp peak at pH 5.6 with an overall recovery of 45% of the starting activity. Preparations of "young and "old" enzymes mixed together also ran as a single band identical with the individual enzymes. No differences in properties resulted when homogenates were prepared in the presence of the protease inhibitor, toluenesulfonyl fluoride. Clear proof that the differences in behavior are not due to protease activity is found in the fact that the respective N- and C-terminal groups remained unchanged. Thus, no N-terminal amino acid could be detected for either nematode enolase. This result is presumably due to the presence of an acetylated N-terminal amino acid. *N*-Acetylalanine has been found in the N-terminal position of rabbit muscle enolase (Winstead & Wold, 1964) and is blocked in enolase from two species of salmon (Ruth et al., 1970). The C-terminal group was found to be leucine for both "young" and "old" enolase. Other amino acids released from the C-terminal end of both proteins are valine and isoleucine.

TABLE II: Spectral Properties of "Young" and "Old" Enolase.

	A_{280}/mg	A_{max}	A_{min}	R(280/260)
enzyme in buffer[a]				
young	0.6	280	252.5	1.9 ± 0.1
old	0.8–1.0	278	250	1.3 ± 0.2
enzyme in 6 M Gdn·HCl				
young	0.79	277	250	1.3 ± 0.05
old	0.76	277	250	1.4 ± 0.05

[a] Dialyzed overnight against 10 mM Tris-HCl containing 1 mM $MgSO_4$.

From Table I, it can be seen that, within the accuracy of the determinations, there is no significant difference in amino acid composition between "young" and "old" enolase. Small changes would not, of course, be detectable.

The altered properties of the "old" enzyme are not a result of changes in S–S bonding. The titration of -SH groups in "young" and "old" enzyme yields identical results. In both cases, two -SH groups appear to be normally available. In the presence of 0.5% $NaDodSO_4$, two additional groups can be titrated. Total -SH value is 3.5 residues. Since there are four cysteine residues in the enzyme (Table I), all the -SH groups appear to exist in the reduced form in both enzymes.

Table II shows the spectral properties of "young" and "old" enolase. The latter shows a substantially greater absorbance/mg at 280 nm than the "young" enzyme. The 280/260 ratios also differ considerably, being 1.9 and 1.3, respectively. Equal mixtures of "young" and "old" enolase show an intermediate value of 1.65. These results were consistently observed using many "young" and "old" preparations. Since tryptophan and tyrosine are present in equal amounts in both "young" and "old" enolase (Table I), the hyperchromic effect noted above must be due to changes in the conformation of the enzyme in which additional chromophoric groups become exposed. In 6 M Gdn·HCl, the spectral differences disappear (Table II).

Support for the idea of differential exposure of certain amino acid residues is obtained from the titration of tryptophan. From Table III, it can be seen that in comparison with "young" enolase, the "old" preparation has one extra tryptophan residue exposed to the medium. In the "young" enolase, an equivalent value is reached only when urea is added. Both enzyme preparations ultimately reach the same value (6.5 and 7.0 tryptophan residues) after treatment with 8 M urea when the chains are presumably completely unfolded.

Titration of the hydroxyl group of tyrosine (Figure 1) shows that, in the "old" enzyme, two extra residues are exposed at all pH values above pH 9.7. In the presence of 6.0 M Gdn·HCl (pH 12.5), both enzymes show the same number of residues, namely, 16.9 and 16.5 mol/mol of protein, respectively, for "young" and "old" preparations. Under these conditions, the molar extinction coefficient was also the same for both en-

TABLE III: Titration of Tryptophan in "Old" and "Young" Enolase.[a]

urea concn	mol of tryptophan residues per mol of enolase[b]	
	old	young
no urea	3.5	2.4
0.8 M	3.5	3.7
1.6 M	3.5	3.7
2.4 M	5.2	4.6
4.0 M	4.9	4.6
8.0 M	6.5	7.03[c]

[a] Desalted protein (typically around 0.6 mg) was used in the titration. The details of the experiments are given in the text. [b] Based on a molecular weight of 82 000. Protein determinations by ninhydrin method. [c] Protein determined by direct weighing.

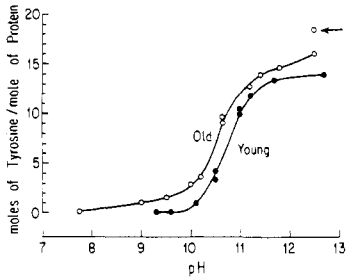

FIGURE 1: Titration of tyrosine in "young" and "old" enolase. (O—O) "Old" enzyme; (●—●) "young" enzyme. Arrow indicates the value obtained for both "young" and "old" enolase in 6 M guanidine.

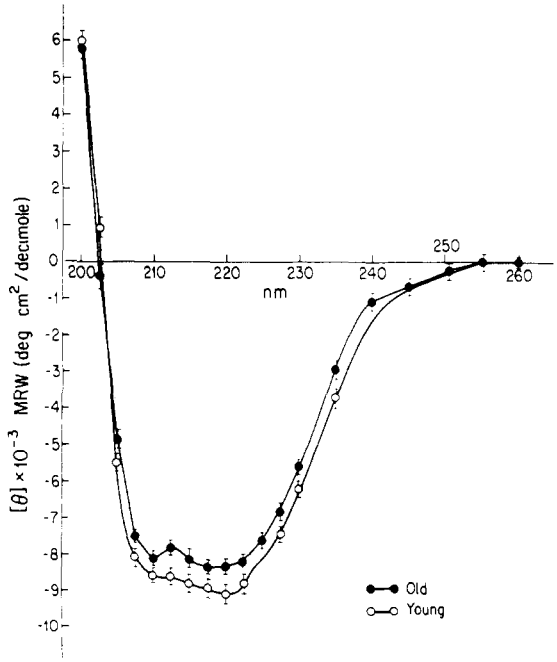

FIGURE 2: Circular dichroism spectra of "young" and "old" enolase in far UV. (O—O) "Young" enzyme; (●—●) "old" enzyme. The bars represent the magnitude of the variation in the repeated scans in two different preparations.

zymes, confirming that there are no differences in the total number of aromatic residues.

Circular dichroism spectra (Figure 2) indicate that there are differences in the secondary structure of enolase derived from "young" and "old" animals. The spectra obtained in the far-ultraviolet region of 260 to 200 nm show negative bands

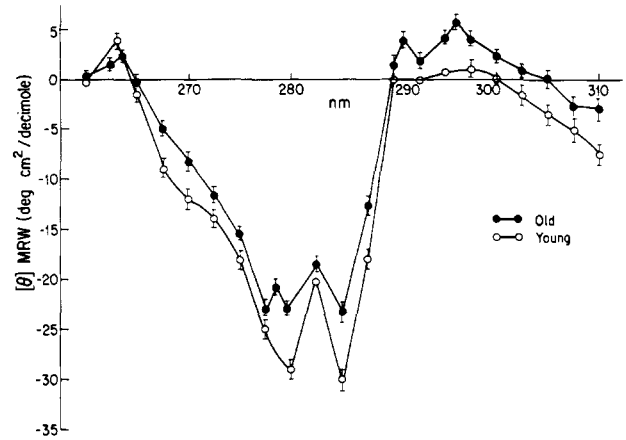

FIGURE 3: Circular dichroism spectra of "young" and "old" enolase in near UV. (O—O) "Young" enzyme; (●—●) "old" enzyme. The bars represent the magnitude of the variation in the repeated scans in two different preparations.

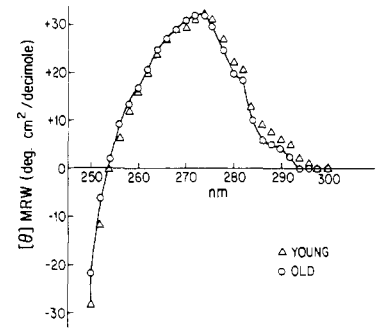

FIGURE 4: Circular dichroism spectra of "young" and "old" enolase in 6 M guanidine hydrochloride. (O—O) "Young" enzyme; (△—△) "old" enzyme.

near 210–220 nm for both the "young" and "old" enzymes. However, the mean residue ellipticities at the negative minimum at 210 and 220 nm are respectively −8114 and −8309 deg cm^{-2} $dmol^{-1}$ for "old" enolase, whereas these values are significantly higher (−8673 and −9191 deg cm^{-1} $dmol^{-1}$, respectively) for "young" enolase. The position and shape of these bands indicate the presence of α-helical forms as described by Sarker & Doty (1966). The spectra show changes in the amounts of α-helical, β-sheet, and unordered coiled structures.

The near-UV region from 310 to 260 nm (Figure 3) shows positive and negative CD bands. The spectra are complex and presumably are originated from overlapping of the CD spectra of aromatic amino acid residues. The peak at 280 nm, however, can be attributed to tyrosine and that at 296 nm to tryptophan. The differences in spectra obtained for the "young" and "old" enolase clearly indicate different electronic environments experienced by aromatic residues in these two enzymes.

In 6 M Gdn·HCl, when both enzymes are unfolded, the differences in the CD spectrum disappear (Figure 4). This result suggests that there is no sequence change around the aromatic amino acids, though it does not prove the case.

The circular dichroism spectra confirm the results obtained by titration of tyrosine hydroxyl groups and tryptophan residues. One may conclude that the aromatic groups on the protein structure exist under different environmental conditions in "young" vs. "old" enolase.

Further clear evidence of changes in properties is found in the different rate of action of trypsin on "young" and "old"

TABLE IV: Recovery of "Young" and "Old" Enolase from Columns of DE-52.[a]

	young				old			
Passage	starting sp act. (U/mg)	% recovery	cumulative % recovered	recovered sp act. (U/mg)	starting sp act. (U/mg)	% recovery	cumulative % recovered	recovered sp act. (U/mg)
I	1131	87	87	1083	786	52	52	702
II	1083	91	79	723	702	41	21	702
III	723	76	60	721	702	0.0	0	

[a] The DE-52 was prepared according to procedure B in Materials and Methods. Reloading of the protein onto subsequent columns was made at a protein concentration of 0.5–0.6 mg/mL.

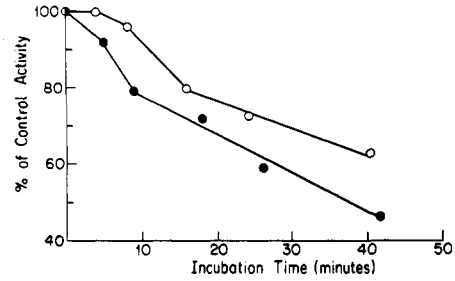

FIGURE 5: Inactivation of enolase by trypsin. (O—O) "Young" enolase; (●—●) "old" enolase. In the control experiment (enzyme with no trypsin present), there was no significant change in activity over the indicated time period.

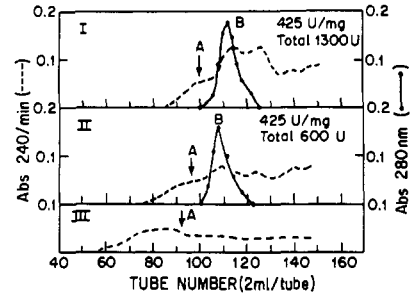

FIGURE 6: Repeated chromatography of "old" enolase on DE-52 (O—O) enzyme activity; (- - -) protein. Diagrams I, II, and III represent sequential passes of "old" enolase through a column of DE-52. Peak B represents recovered enolase in each case. Peak A is inactive enolase which can be detected on immunodiffusion plates. The specific activity of the enzyme recovered in I (peak B) was 425 units/mg. After repassage, the recovered enzyme in II still had the same specific activity. However, the total activity dropped from 1300 units to 600 units.

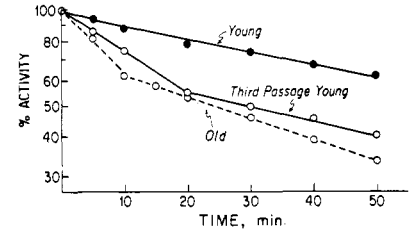

FIGURE 7: Heat stability of "young" enolase after treatment on DE-52. (●—●) Original "young" enolase; (O—O) "young" enolase after three passages through DE-52; (O - - - O) "old" enolase. The temperature was 52.5 °C in all cases.

enolase. As can be seen from Figure 5, the "old" enzyme is the more sensitive of the two. Trypsin attacks proteins at basic amino acid residues (lysine and arginine). Since electrofocusing shows that there is no increase in the number of basic amino acids, this experiment, too, affirms that the conformation of "old" enolase is altered, thus permitting the more effective attack of trypsin on the molecule.

When "young" enzyme is rechromatographed repeatedly on a column of DE-52 equilibrated as in procedure A (see Materials and Methods), loss of activity is very small for the first two runs and then increases to 25–30% on the third passage through the column. When "old" enolase (pure enzyme, as judged by immunodiffusion and gel electrophoresis) is treated by the same procedure, over 50% of the total activity is lost with the first passage and the material is in part converted to an inactive protein (Figure 6, peak A). This inactive material reacts with antiserum prepared to pure "young" or "old" enolase. Other fractions (except for enolase itself) do not react with the antiserum. It is important to note that the specific activity of the recovered "old" enolase is unchanged. When this recovered enzyme (Figure 6, peak B) is rechromatographed on the same type of column, a further 50% of the remaining enzyme activity is lost and more inactive protein appears (Figure 6, II). Again, the specific activity of the recovered enzyme is unchanged. By the third passage, no enzyme activity remains (Figure 6, III). It is clear that, each time it is chromatographed, part of the pure "old" enolase is being converted to the antiserum-reacting material in peak A. The latter did not preexist as an impurity in the "old" enolase. If it did, the specific activity of the recovered enzyme would have increased with the removal of the inactive protein. Since the material in peak A reacts with the antiserum prepared to "young" or "old" enolase, it is designated "inactive enolase."

From the above results, it can be seen that another difference between "young" and "old" enolase is the instability of the latter to ion-exchange chromatography on DE-52.

Table IV shows the results of repeated chromatography of "young" vs. "old" enolase on DE-52 (prepared according to procedure B). The "old" enolase continues to exhibit unchanged specific activity but a large proportion of the enzyme is lost after each passage through the column. On the other hand, little "young" enzyme is lost in the first two passages. However, the specific activity drops sharply after the second passage, and the loss subsequently increases. After the third passage the product exhibits a biphasic heat-sensitivity curve (Figure 7). It also exhibits some of the spectral properties of "old" enzyme shown in Table II: A_{280}/mg, 0.6; A_{max}, 280 nm; A_{min}, 250 nm; R (280/260), 1.3. The last value clearly indicates a change in the conformation of aromatic groups. Moreover, thrice-columned "young" enolase gives rise to an inactive enolase: the combined fractions which come after the peak of enzyme activity give a line of identity with inactive enolase obtained from "old" enolase, using antiserum prepared to "young" or "old" enolase (Figure 8). Antiserum prepared to this thrice-columned "young" enolase has a greater affinity for "old" enolase than for "young" enolase.[3] These experiments

[3] H. K. Sharma & M. Rothstein, in preparation.

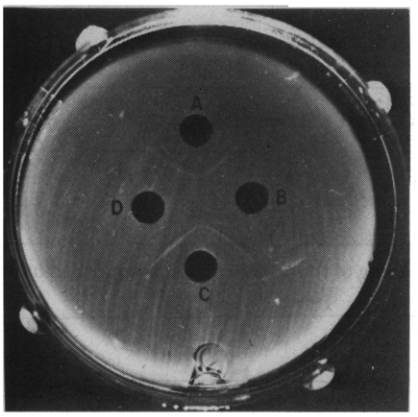

FIGURE 8: Immunodiffusion of inactive enolase. (A) Antiserum (20 μL) prepared to "young" enolase; (B) inactive enolase (3–5 μg) isolated during purification of "old" enolase; (C) antiserum (20 μL) prepared to "old" enolase; (D) inactive enolase (3–5 μg) obtained by passing "young" enolase thrice through a column of DE-52.

show that "young" enolase can be converted to a product with properties at least superficially similar to those of "old" enolase.

It should be noted that the position of "inactive" enolase seems to depend upon the degree of equilibration of the DE-52. If washing of the DE-52 with Tris buffer is carried out for 3–4 h (procedure A), inactive enolase consistently appears before the enolase peak; if the DE-52 is equilibrated more thoroughly (procedure B), the same losses in "old" enzyme occur but the inactive enolase usually, though not always, elutes just after the enolase peak. From Figure 6, it can be seen that, in addition to inactive enolase, "old" enolase is converted to protein which elutes in a generalized manner. None of this protein reacts with the enolase antibodies except for the material in the areas representing enolase or inactive enolase.

Inactive enolase can be readily identified on immunodiffusion plates. Though it reacts with antiserum prepared to pure "young" or "old" enolase, it yields a recognizable pattern of partial identity with the latter. The results obtained in the immunological experiments will be published separately. The formation of this distinctive precipitation line made it possible to detect inactive enolase in homogenates of old *T. aceti* (Sharma et al., 1976). Thus, the material is not simply an artifact of the column procedure but occurs naturally, perhaps as a result of the in vivo denaturation of the less stable "old" enolase. Inactive enolase is absent from similar preparations from young organisms (Sharma et al., 1976).

As would be expected, inactive enolase is indistinguishable from "young" or "old" enolase on $NaDodSO_4$ gels. On gel electrophoresis, it moves slightly less toward the anode than does "young" (or "old") enolase. Therefore, inactive enolase is not formed by deamidation. If such were the case, it would move closer to the anode, not farther.

Discussion

The cause of the age-related changes in enolase and other enzymes in nematodes (Reiss & Rothstein, 1975; Gupta & Rothstein, 1976a; Reznick & Gershon, 1977; Sharma et al., 1976), rats (Reiss & Gershon, 1976), and mice (Gershon & Gershon, 1973) has not yet been determined. In fact, what does not happen in the alteration of "old" enzymes is more clear than what does occur. There appear to be no gross changes in amino acid composition, nor any substitutions which result in a change of charge. Beyond any reasonable doubt, proteolysis is not a factor. No proteolytic cuts occur insofar as these might change the molecular weight, charge, or ability of the enzymes to react with antibodies. These observations hold true for all four altered enzymes thus far purified (Reiss & Gershon, 1976; Reiss & Rothstein, 1975; Gupta & Rothstein, 1976a; Reznick & Gershon, 1977). Mixing of "young" and "old" homogenates (Gershon & Gershon, 1973) or allowing crude homogenates to stand for several hours (Reiss & Rothstein, 1975) does not change the properties of the various enzymes so far studied. Similarly, the presence or absence of protease inhibitors does not affect the respective properties of "young" or "old" enolase (see Results) or isocitrate lyase (Reiss & Rothstein, 1975). Most persuasive is the present finding that the respective N-terminal and C-terminal groups are unchanged in "young" and "old" enolase.

Acylation, phosphorylation, or dephosphorylation should not be involved in the formation of "old" enzymes, as a change in electrophoretic mobility would result. Such a change could not be detected in enolase or in phosphoglycerate kinase (Gupta & Rothstein, 1976a), a relatively small protein possessing a single chain. Goren et al. (1977) found no differences in the isoelectric properties of "young" and "old" aldolase from nematodes or superoxide dismutase from rat liver. By contrast, loss of a single amide group in one of four protein chains is readily detectable in aldolase (Gürtler & Leuthardt, 1970). Methylation seems unlikely, as methylated derivatives of lysine were not observed in the amino acid analyses of enolase or phosphoglycerate kinase. The presence of isozymes cannot account for the results as neither enolase (Sharma et al., 1976) nor phosphoglycerate kinase (Gupta & Rothstein, 1976a) from *T. aceti* possesses isozymic forms. Furthermore, the altered properties of "old" enolase are not due to oxidation of –SH groups: the cysteine residues of both "young" and "old" enolase are all in the reduced form and are equal in number.

If the alteration of "old" enzymes is due solely to a postsynthetic modification, as proposed by Reiss & Rothstein (1974) and Rothstein (1977), then the change in protein conformation may result from an enzyme-mediated action, a kinetically determined change in the folding of the protein or passive accumulation (or removal) of some small component which can affect the function of the enzyme. The results reported in this paper prove that there is a conformational difference between "young" and "old" enolase. Moreover, there is strongly suggestive evidence that this conformational change alone is responsible for the altered properties of the latter. The hyperchromicity exhibited by "old" enolase (Table II) compared with the "young" enzyme is not due to the presence of extra tyrosine, tryptophan, or –SH groups. Moreover, the differences disappear in 6 M guanidine. This increased absorbance must therefore be caused by a difference in the conformation of the "old" forms of the enzyme. This concept is clearly borne out by the results of the titrations of tryptophan and tyrosine (Table III and Figure 1) and the results of the CD studies (Figures 2–4). The differential inactivation of "young" and "old" enolase by trypsin (Figure 5) adds one more characteristic in which the two forms of the enzyme differ.

Rotman (1970) reported that individual molecules of β-galactosidase from *E. coli*, after prolonged storage in a freezer or refrigerator, displayed a wide range of activities from 5 to 100% of normal, whereas, in the freshly prepared samples, variation of individual molecules was within 10%. The results provide evidence that individual β-galactosidase molecules can be "altered" and yet remain partly active without any considerations extrinsic to the enzyme. Similar conclusions can be drawn from the results presented in this paper. The data make it explicit that "young" enolase can be converted in vitro at least partly into a new form of the enzyme which possesses

several characteristics similar to those of "old" enolase. After three passes through DE-52, the "young" enolase shows a reduction in specific activity to the level of "old" enzyme, a biphasic, though not identical, heat-sensitivity curve, a preferred reaction of its antiserum with "old" enolase and formation of an "inactive" enolase which is immunologically identical with inactive enolase formed from the "old" enzyme. Clearly, this alteration process occurs without any change of sequence. Though these results do not prove that "old" enzymes are simply conformationally altered "young" enzymes, they certainly prove that such a conversion is possible. The fact that the respective UV and CD spectra of "young" and "old" enolase in 6 M guanidine are identical provides strong evidence for this hypothesis.

An alternative is that the demonstrated conformational change in "old" enzymes is brought about by an amino acid substitution which does not alter the charge, for example, alanine for valine. Such a change could readily escape detection in a total amino acid analysis. It is well established that changing a single amino acid residue can have widely varying effects, from inactivating an enzyme to producing little change. For example, substitution of a single neutral amino acid for another has been suggested as the cause for complete inactivation of galactose-1-phosphate uridyltransferase in human red blood cells (Dale & Popjak, 1976) giving rise to galactosemia. A single amino acid substitution (but with a change of charge) in hemoglobin leads to a partly functional but defective molecule (sickle cell anemia). On the other hand, many single changes in hemoglobin sequence are known to be innocuous in terms of function. At the present time, there is no evidence either for or against the idea of substituting an amino acid for one of like charge to explain the age-related alteration of enzymes. Still, to obtain such an effect, one would have to postulate rather specific age-related changes in parts of the protein-synthesizing system in a wide variety of organisms.

Another explanation for altered enzymes could lie in the removal of some noncharged moiety from the proteins—perhaps a lipid or glycosyl group. Addition of such a component is not likely, as the in vitro conversions cannot be explained in this way. If removal of such components occurs, it would require the increasing effectiveness of appropriate deglycosylating or delipidating enzymes in old organisms of widely diverse species. Moreover, by chance, exactly the same process would have to occur nonenzymatically in the in vitro experiments. In brief, the formation of altered (old) enzymes by removal of noncharged moieties is a possible but rather strained explanation of the phenomenon of "old" enzymes.

The heat-stability diagram (Figure 7) shows that three enzymatically active forms of nematode enolase exist. These are, in order of decreasing stability: "young" (I); "stable old" (II); and "unstable old" enolase (III). This information makes it tempting to suggest that the following set of conversions represents the sequence of enzyme alterations which occurs in vivo in old animals: "young" enolase → stable "old" enolase → unstable "old" enolase → inactive enolase. The demonstrated in vitro conversion of "old" enolase to immunoreactive but enzymatically inactive enolase and the discovery that the latter is present in homogenates of old *T. aceti* provides strong evidence for the last step. The demonstrated conversion in vitro of "young" enolase to a protein similar in properties to "old" enolase and detection of an immunologically identical inactive enolase from both enzymes provides support for the first and subsequent steps.

The reason for the accumulation of altered enzymes in old organisms may lie in protein turnover. If protein turnover slows in old animals, the "dwell time" of each enzyme in the cell would increase and altered enzymes would accumulate instead of being rapidly replaced with newly synthesized molecules. The rate of accumulation and the nature of the intermediates would depend on the native stability of each enzyme. This hypothesis can explain all the results so far obtained with altered enzymes (Rothstein, 1977). Zeelon et al. (1973) report that the half-life of a nematode aldolase increases from 40 h in 7-day old (young) *T. aceti* to 200 h in 24-day old (old) organisms. Prasanna & Lane (1977) reported that the incorporation of labeled methionine into protein declined dramatically with age in *T. aceti*. These authors also find a dramatic increase in the half-life of soluble nematode proteins from 30 h for young to 250 h for old organisms (private communication). Unfortunately, there is little other applicable data available at this time on the half-lives of proteins in old animals.

The data presented here support the idea that the age-related changes found in the properties of "old" enolase are a result of conformational changes arising from a subtle and irreversible denaturation of the enzyme without sequence changes. However, other posttranslational mechanisms or sequence changes could be responsible. Final solution to the problem must await the detailed sequence analysis of "young" and "old" proteins.

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References

Airhart, J., Sibiga, S., Sanders, H., & Khairallah, E. A. (1973) *Anal. Biochem. 53,* 132–140.

Ambler, R. P. (1967) *Methods Enzymol. 11,* 155–166.

Dale, G. L., & Popjak, G. (1976) *J. Biol. Chem. 251,* 1057–1063.

Donovan, J. W.(1973) *Methods Enzymol. 27,* 525–548.

Gershon, H., & Gershon, D. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70,* 909–913.

Goren, P., Reznick, A. Z., Reiss, U., & Gershon, D. (1977) *FEBS Lett. 84,* 83–86.

Gros, C., & Labouesse, B. (1969) *Eur. J. Biochem. 7,* 463–470.

Gupta, S. K., & Rothstein, M. (1976a) *Biochim. Biophys. Acta 445,* 632–644.

Gupta, S.K., & Rothstein, M. (1976b) *Arch. Biochem. Biophys. 174,* 333–338.

Gürtler, B., & Leuthardt, F. (1970) *Helv. Chim. Acta 53,* 654–658.

Habeeb, A. F. S. A. (1972) *Methods Enzymol. 25,* 457–464.

Hirs, C. H. W.(1967a) *Methods Enzymol. 11,* 197–199.

Hirs, C. H. W. (1967b) *Methods Enzymol. 11,* 325–329.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193,* 265–275.

Prasanna, H. R., & Lane, R. S. (1977) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36,* 292.

Reiss, U., & Gershon, D. (1976) *Eur. J. Biochem. 63,* 617–623.

Reiss, U., & Rothstein, M. (1974) *Biochem. Biophys. Res. Commun. 61,* 1012–1016.

Reiss, U., & Rothstein, M. (1975) *J. Biol. Chem. 250,* 826–830.

Reznick, A. Z., & Gershon, D. (1977) *Mech. Ageing Dev. 6,* 345–353.

Rothstein, M. (1975) *Mech. Ageing Dev. 4,* 325–338.

Rothstein, M. (1977) *Mech. Ageing Dev. 6,* 241-257.
Rotman, M. B. (1970) in *The Lactose Operon* (Beckwith & Zipser, Eds.) pp 279-289, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
Ruth, R. C., Soja, D. M., & Wold, F. (1970) *Arch. Biochem. Biophys. 140,* 1-10.
Sage, H. J., & Singer, S. J. (1962) *Biochemistry 1,* 305-317.
Sarkar, P. K., & Doty, P. (1966) *Proc. Natl. Acad. Sci. U.S.A. 55,* 981-989.
Sedlak, J., & Lindsay, R. H. (1968) *Anal. Biochem. 25,* 192-205.
Sharma, H. K., Gupta, S. K., & Rothstein, M. (1976) *Arch. Biochem. Biophys. 174,* 324-332.
Spande, T. F., & Witkop, B. (1967) *Methods Enzymol. 11,* 498-506.
Steinhagen-Thiessen, E., & Hilz, H. (1976) *Mech. Ageing Dev. 5,* 447-457.
Townend, R., Kumosinski, T. F., Timasheff, S. N., Fasman, G. D., & Davidson, B. (1966) *Biochem. Biophys. Res. Commun. 23,* 163-169.
Weber, A., Gregori, C., & Schapira, F. (1976) *Biochim. Biophys. Acta 444,* 810-815.
Weiner, A. M., Platt, T., & Weber, K. (1972) *J. Biol. Chem. 247,* 3242-3251.
Winstead, J. A., & Wold, F. (1964) *Biochemistry 3,* 791-795.
Wrigley, C. W. (1971) *Methods Enzymol. 22,* 559-564.
Zeelon, P., Gershon, H., & Gershon, D. (1973) *Biochemistry 12,* 1743-1750.

Epoxide Hydrase: Structure–Activity Relationships of in Vitro Stimulators of the Microsomal Activity†

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ABSTRACT: Benzophenone, 9-fluorenone, diphenylcyclopropenone, benzyl phenyl ketone, propyl phenyl ketone, chalcone, and chalcone oxide are in vitro stimulators of the activity of microsomal epoxide hydrase (EC 4.2.1.63). Chalcone oxide and 9-fluorenone, the more effective of these stimulators, were found to be more effective than metyrapone, a previously reported stimulator of this enzyme. Other aryl ketones, such as acetophenone, benzyl ethyl ketone, dibenzyl ketone, and phenylmethylcylopropenone, do not stimulate microsomal epoxide hydrase activity. Kinetic analyses established that, while chalcone, 9-fluorenone, diphenylcyclopropenone, and chalcone oxide all increase the observed V_{max} of the hydrase reaction, diphenylcyclopropenone and chalcone epoxide increase V_{max} and K_M by comparable factors. At higher concentrations of the styrene oxide, substrate inhibition is observed. The observed kinetics of microsomal epoxide hydrase with styrene oxide as substrate are consistent with the formation of an inactive ES_2 complex at high substrate concentrations. One in vitro effect of chalcone oxide is to largely eliminate the inhibition caused by high styrene oxide concentrations. It is concluded that compounds containing an aryl carbonyl substituted with an additional hydrophobic group are effective in vitro stimulators of epoxide hydrase. The data suggest that such compounds bind at a site which is distinct from the catalytic site where the styrene oxide substrate binds.

It is now accepted that most chemical carcinogens must be metabolically converted into reactive, electrophilic derivatives before they are capable of initiating tumors. In particular, it is believed that the carcinogenicity and mutagenicity of polycyclic aromatic hydrocarbons such as benzo[*a*]pyrene are the result of in vivo metabolism which produces reactive intermediates (ultimate carcinogens) that bind covalently with macromolecules within the cells (Miller & Miller, 1966; Sims & Grover, 1974; Jerina & Daly, 1974; Heidelberger, 1975). Recent experimental evidence, involving investigations of both the binding of benzo[*a*]pyrene derivatives to DNA and the relative mutagenicity of benzo[*a*]pyrene derivatives, indicates that diol epoxide derivatives (7,8-dihydro-7,8-dihydroxybenzo[*a*]pyrene 9,10-oxides) function as major ultimate carcinogenic metabolites of this polycyclic hydrocarbon (Thakker et al., 1977a, and references therein; Yang et al., 1977b, and references therein). The recent investigations have also established that the metabolic conversion of benzo[*a*]pyrene to the reactive diol epoxide derivatives requires microsomal monooxygenase (EC 1.14.14.1) and microsomal epoxide hydrase (EC 4.2.1.63) activities (Booth & Sims, 1976; Yang et al., 1976, 1977a, b; Thakker et al., 1976, 1977a).

The properties of epoxide hydrase have been the subject of several investigations (for a review of the early work, see Oesch, 1972). Recognition that epoxide hydrase is involved in the conversion of carcinogenic polycyclic hydrocarbons such as benzo[*a*]pyrene into their ultimate carcinogenic forms has led to increased interest in this enzyme. Recent papers, for example, have described additional investigations of the substrate specificity of both membrane-bound and purified epoxide hydrase (Bentley et al., 1976; Lu et al., 1977), a simplified method of purifying microsomal epoxide hydrase (Knowles & Burchell, 1977), new, sensitive assay procedures (Schmassmann et al., 1976; Jerina et al., 1977), additional information regarding the mechanism of the enzymatic ca-

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