Altered enolase in aged *Turbatrix aceti* results from conformational changes in the enzyme
(altered enzymes/age-related changes/aging nematodes/unfolding and folding/2-phospho-D-glycerate hydrolyase)

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**ABSTRACT** Young- and old-type enolases (2-phospho-D-glycerate hydrolyase, EC 4.2.1.11) from the free-living nematode *Turbatrix aceti* can be unfolded in 1.25 M guanidine hydrochloride and subsequently refolded with essentially a quantitative recovery. After refolding, both enolases form an identical or near-identical third type of the enzyme as determined by spectral criteria, sensitivity to heat, immunotitration, and rate of inactivation by bacterial protease. By the same criteria, the refolded enolase is closer in conformation to the native old form of the enzyme than to the young form. The results prove that young and old enolases are conformational isomers and that an in vivo transformation from young to old enzyme takes place by conformational changes without covalent modification. The process may be related to the previously demonstrated slowing of enolase turnover in *T. aceti*. Errors in sequence cannot be involved in the age-related alteration of the enzyme.

A number of altered enzymes have been isolated from aged animals. Included are isocitrate lyase (1), phosphoglycerate kinase (2), and enolase (8) from the free-living nematode *Turbatrix aceti*; superoxide dismutase (4) from rat liver; and phosphoglycerate kinase (5) from rat muscle.

In theory, enzymes may become "altered" by either errors in sequence or modification after synthesis. A great deal of evidence has been accumulated that makes the former mechanism highly unlikely. For example, none of the altered enzymes show changes in charge; this narrowly restricts the kinds of amino acid substitutions that could occur. Moreover, several enzymes have been reported to be unaltered in old animals (6-9), and even very old subjects synthesize normal enzymes in blood tissues (10). It is difficult to imagine how substantial errors in synthesis could occur in some proteins but not others. On the other hand, Sharma and Rothstein (11) have provided substantial evidence that modification involving only a change in conformation after synthesis is responsible for the observed age-related alteration of enolase. In this regard, spectral differences in "young" and "old" enolases disappear in solutions of 6 M guanidine hydrochloride. Moreover, young enolase can be converted to a product similar to old enolase by repeated passage of the former through an ion-exchange column (11). In the process, some of the enolase denatures to an inactive form that is immunologically identical with an inactive product isolated during preparation of the old enzyme (11). Moreover, this inactive enolase has been shown to accumulate naturally in aging *T. aceti* (12). In short, the various forms of enolase appear to be related to one another without covalent differences.

Rothstein and coworkers (12-15) proposed that the conformational changes could be brought about by a slowing of protein turnover. In this way, the "dwell time" of the enzymes in the tissues would increase, resulting in the accumulation of slightly denatured molecules instead of a normal, rapid replacement. Indeed, protein turnover was shown to slow dramatically with age in *T. aceti* (16, 17), although data are lacking on the situation in higher animals.

Although the results describe above lend strong support to the idea of postsynthesis modifications rather than sequence changes as the cause of altered enzymes, unequivocal proof is lacking. In this paper, we provide data that support the idea that old enolase and young enolase from *T. aceti* differ only in conformation. When either type of molecule is unfolded in solutions of guanidine hydrochloride and then permitted to refold, the products are identical or nearly so as judged by spectra, immunological response, sensitivity to heat, and rate of inactivation by protease. Enolases from young and old *T. aceti* showed substantial differences in these characteristics before the unfolding/refolding treatment.

**METHODS AND MATERIALS**

Axenic cultures of young and old *T. aceti* and the purification and estimation of enolase have been described (3). Protein was assayed by the procedure of Lowry et al. (18) and by absorbance at 280 nm. Results are consistent with previous work in which protein concentration was also related to dry weight and a ninhydrin/alkaline hydrolysis procedure (11). All reagents were of analytical grade. Glass-distilled, ion-free water was used throughout. Guanidine hydrochloride was purchased from Pierce. Stock solutions of pure enolase (4-5 mg/ml) were dialyzed overnight against renaturation buffer (5 mM Tris/1 mM EDTA/10 mM MgSO<sub>4</sub>/20 mM KCl/10 mM dithiothreitol, pH 7.5). The dialyzed product was centrifuged at 48,000 X g for 20 min to remove occasional traces of precipitate, and the supernatant solution was used for the studies.

**Denaturation and Renaturation Studies.** The solution of enolase (50 μl; 4.5 mg/ml) was added to a cold, freshly prepared solution of guanidine hydrochloride (2.5 M) in 50 μl of renaturation buffer. The mixture was stirred gently and incubated at 0°C. To determine the rate of loss of enzyme activity, 5-μl samples were withdrawn at appropriate times, diluted in 1 ml of renaturation buffer, and assayed within 30 sec of withdrawal. The assay is rapid and therefore does not affect the rates shown for denaturation and renaturation. Time periods of up to 48 hr and concentrations of guanidine hydrochloride up to 6 M were also tested.

Renaturation was carried out by diluting the treated enzyme 1:200 in renaturation buffer at room temperature and measuring the recovered activity at appropriate times. At 0°C, recovery was extremely slow. At the enzyme concentrations used, enolase precipitated from solutions of guanidine hydrochloride at concentrations of 0.5 M or lower. Therefore, the reagent was not removed by dialysis without prior dilution.

For large-scale preparations, enolase (15-20 mg) was dena-
tured with guanidine hydrochloride (final concentration, 1.25–2.0 M) at 0°C. After 15–30 min, the reaction mixture was diluted 1:200 by mixing with renaturation buffer and incubated in the dark at room temperature under nitrogen. Enolase was assayed as described above at various time intervals, and the enzyme was concentrated on a PM-10 membrane after maximal enzymatic activity was obtained.

**Thermal Inactivation.** The enzyme solution (100 µl; 0.15 mg/ml) was heated at 59°C in a water bath. At various time intervals, 10-µl aliquots were withdrawn and assayed.

**Inactivation by Protease.** The enzyme (100 µl; 0.26 mg/ml) was incubated at 50°C with 15 µl of protease (Bacillus subtilis, type VII, Sigma) solution (2 mg/ml) in renaturation buffer. Aliquots (10 µl) were withdrawn at various time intervals and assayed for enolase activity.

**Circular Dichroism.** Circular dichroism spectra of enolase were recorded at room temperature in 0.05 M phosphate buffer (pH 7.0) with a JASCO Spectropolarimeter, model J-41C. All results are reported as mean residue ellipticity in deg cm²/dmol (mean residue weight of 98). Protein samples (0.5–1.0 mg/ml) were dialyzed overnight at room temperature against phosphate buffer and centrifuged to remove any precipitate. A protein concentration of 0.4–0.5 mg/ml and a cell of 0.1-cm pathlength were used. The baseline was recorded at the beginning and the end of each experiment.

**Spectral Studies.** The absorbance values for the various forms of enolase at 280 nm were obtained at room temperature in a Cary 15 spectrophotometer. Enzyme preparations were dialyzed overnight against freshly prepared renaturation buffer and were centrifuged. Protein concentrations were typically 1–2 mg/ml in the various studies.

**Difference spectra were determined at a protein concentration of 4.5 mg/ml in a partitioned cell of 1.0-cm pathlength at 22°C in a Cary 15 spectrophotometer. A baseline (290–330 nm) was recorded with 0.8 ml of enzyme solution in one chamber and 0.8 ml of guanidine hydrochloride solution in the other chamber. The contents of the experimental cells were mixed and the spectrum was scanned at various time intervals. The number of tryptophan molecules displaced was calculated by the procedure of Edelhoch (19).

**Immunotitration.** A monospecific antiserum against young enolase was raised and purified as described (12, 16). Immunotitration of enolase was performed by adding increasing amounts of antiserum to tubes containing 12.3 µg of protein in 200 µl of renaturation buffer. The reaction mixture was incubated overnight at 4°C. It was then centrifuged at 6000 × g for 10 min, and enolase activity in the supernatant was determined. Experiments using preimmune antiserum served as a control.

**RESULTS**

Compared with old enolase, young enzyme denatured at a faster rate (Fig. 1). Renaturation of the unfolded enolase occurred quickly and reached completion in approximately 60 min in both cases. There was a complete recovery of the activity with old enzyme, whereas it varied from 65% to 85% in the case of young preparations. The specific activity of the young enzyme was 1100–1200 units/mg and that of the refolded enzyme was 810 units/mg. Therefore, the recovery of 65–85% of the starting activity represented close to 100% conversion of young molecules to renatured molecules. That is, each molecule of enzyme that started with an activity of 1100 units/mg converted to a molecule having an activity of only 810 units/mg. Because old enzyme started at 800 units/mg, total activity should not have changed after refolding if no loss of active enzyme molecules occurred; indeed, recovery was 100%.

**Fig. 1.** Rate of denaturation and renaturation of enolase. Old enolase denaturation (○) and renaturation (●); young enolase denaturation (□) and renaturation (●). Denaturation was performed in 1.25 M guanidine hydrochloride.

Recovered activity was dependent on the temperature at which denaturation was performed. Above 6°C, yields were reduced. When denaturation was performed at low protein concentration, inconsistent levels of renaturation resulted. Changes of pH between 7.0 and 8.0 during denaturation had no effect on the refolding. The length of the incubation period for denaturation (up to 48 hr) at any concentration of guanidine hydrochloride (up to 6 M) in the denaturation step had no effect on the magnitude of renaturation.

The UV difference spectrum of unfolded and native young enolases exhibited negative and positive absorption in the aromatic regions (Fig. 2A). These effects were produced instantaneously, and their intensity and nature did not change with time. The negative values at 280 and 288 nm corresponded to the displacement of 2.6 mol and 2.5 mol of tryptophan in the young and old enolases, respectively. Clearly, the unfolded young and old enolases have similar structures. The large differences in UV absorption of unfolded proteins disappeared when the enzymes were renatured (Fig. 2B). The results suggest that the renatured forms not only closely resemble each other.
but also are similar to the native old form in spectral properties.

Other properties of the renatured enzymes confirmed these results. The $A_{280}$ values were 4.61 ± 0.1 for native young enolase, 5.55 ± 0.1 for native old enolase, and 5.61 ± 0.18 and 5.31 ± 0.15 for refolded young and old enolase, respectively. The latter values were close to the absorbance of old enzyme. A monophasic rate of heat inactivation has been observed with the young enzyme, whereas the old enzyme had a biphasic rate of inactivation (3). Refolded young and old enzymes showed practically identical biphasic patterns of heat inactivation, although they were different from that of old enolase (Fig. 3). The refolded enzyme patterns were remarkably similar to that obtained after young enolase was altered by repeated chromatography on DEAE-cellulose (DE-52) (11). The rate of inactivation of the various enolase forms by *B. subtilis* protease (Fig. 4) also supported the idea of near-identity for the refolded products. The old enzyme was more susceptible than the young enzyme to protease inactivation. After denaturation, both forms attained an intermediate and nearly identical level. Immunotitration of the various forms of enolase with its monospecific antiserum (prepared against young enzyme) also showed the apparent identity of the refolded enolases (Table 1), which yielded identical values. The refolded enzymes were obviously antigenically similar to the native old enzyme. Further evidence for the similarity of the two refolded enolases was shown by the circular dichroism studies (Fig. 5) in which the refolded forms of young and old enzymes had similar spectra that fell between the respective patterns for young and old enolases.

The above results show that both young and old enolases, after denaturation and renaturation, are identical or closely so, whereas it is well established that the native forms of young and old enolase differ substantially (3, 11). Renatured enolase appears to be closest in conformation to native old enolase. Given the rather extensive treatment involved in isolation and denaturing/renaturing experiments, the results obtained comparing young and old enolases are very much alike—in fact, as close as can be achieved by comparing two different young preparations or two old preparations.

**DISCUSSION**

It is clear from the data that both the young and the old forms of enolase can be unfolded and refolded into the same new structure that is similar to that of the original old enzyme. Therefore, in young and old *T. aceti*, the enzyme must have existed simply as conformational isomers. One may conclude that enolase comes off the ribosomes with young conformation and is subsequently modified to the old form in old organisms. Errors in sequence cannot be involved in the process.

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**Table 1. Immunotitration of enolase with young antiserum**

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<th>50% titer, µl</th>
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<td>Young:</td>
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<td>Native</td>
<td>56</td>
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<td>Refolded</td>
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<td>Native</td>
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<td>Refolded</td>
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* Young antiserum denotes antiserum raised against enolase from young animals; solutions contained 12.3 µg of pure enzyme.

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**Fig. 3.** Thermal inactivation of various forms of enolase at 59°C. ●, Young enolase; △, native old enolase; ○, refolded young enolase; ▲, refolded old enolase.

**Fig. 4.** Effect of *B. subtilis* protease on various forms of enolase. ●, Native young enolase; ○, native old enolase; ▲, refolded young enolase; △, refolded old enolase.

**Fig. 5.** Circular dichroism spectra of native and refolded enolase. ○—○, Native young enolase; ······, refolded young enolase; △—△, native old enolase; ····, refolded old enolase. Protein concentration was 0.4 mg/ml in all preparations. MRW, mean residue weight (the values are negative).
The cause of the conformational changes may be related to a slowed protein turnover in old organisms, as proposed earlier by Rothstein and coworkers (12–15). In support of this thesis, it has been shown in T. aceti that rates of synthesis and degradation for enolase (and for soluble proteins) slow consistently and sharply with age (16). The half-life of enolase (or soluble proteins) in old organisms is very long, being approximately 228 hr. Thus, the enolase molecules remain in the old cells for long periods and would have ample opportunity to become altered in conformation. Moreover, the altered molecules would accumulate rather than be removed. It seems reasonable to assume that the change in conformation is caused by kinetic factors and is not brought about by an “altering” enzyme. In young T. aceti turnover is quite rapid (25 hr at age 2 days) (17), so that the enzyme molecules would undergo frequent replacement and would thus not become altered.

An incidental outcome of the turnover studies (at least in T. aceti) is that enzymes altered in the manner of old enolase do not break down faster than normal proteins as has been documented for proteins with altered sequence or proteins containing amino acid analogs (20).

Altered enzymes have been shown to exist in rodents. Rat liver superoxide dismutase (4) and rat muscle phosphoglycerate kinase (5) have been purified, characterized, and shown to be altered in old animals. Altered mouse liver aldolase has been reported in homogenates from old animals (21), but recently the alteration was attributed to the artifactual loss of a COOH-terminal residue in the old preparations (8). Such a process is not involved in the alteration of rat muscle phosphoglycerate kinase because NH2- and COOH-terminal amino acid residues are the same for both young and old enzymes. Presumably, the mechanism of formation of altered enzymes in higher animals is similar to that in T. aceti. Unfortunately, little information is available regarding the effect of age on protein turnover, especially at the advanced ages (approximately 30 mo) utilized for isolation of the old enzymes. Therefore, the turnover aspect of the hypothesis remains speculative.

The nature of the conformational changes in enolase altered \(\text{in vivo}\) are not known. Obviously they are substantial enough to change the UV spectrum and to cause changes in secondary structure (11). Changes in exposure of aromatic amino acid residues are particularly noticeable. The degree of unfolding that is permissible must be substantial, because even after long exposure to 6 M guanidine hydrochloride the enzyme returns to full activity. Presumably, the degree of unfolding/refolding that creates old enolase \(\text{in vivo}\) does not go quite far enough to result in formation of the \(\text{in vitro}\) form, which is neither young nor old, although similar in behavior to old enzyme. The fact that the enzyme is metastable—that is, it can form conformational isomers—would tend to support the idea of kinetic rather than thermodynamic control of protein folding (22–24).

The ability of a fairly large enzyme that consists of two subunits (40,000 daltons each) to refold in essentially quantitative yield, along with its lack of S—S linkages (11), its stability, its ease of purification, the ease of preparing antiserum to the young and old forms of the enzyme, the ease of identification of the various forms and the existence of yet a fourth (inactive) form of the enzyme (3, 12) all should contribute to the value of nematode enolase as a model enzyme for refolding studies.

The authors wish to note the contribution of Joseph J. LoPresti in helping to establish the conditions for unfolding and refolding the enolase in the early stages of this work. This work was supported in part by Grant AG00618 from the National Institute on Aging.