"Conformational" Isoenzymes of Ascarid Enolase*

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The present studies were initiated to determine the biochemical basis for the existence of multiple, electrophoretically distinct enolase activities in the parasitic round worm Ascaris suum. A total of five activities were resolved by electrophoresis of worm body fluids on cellulose polyacetate strips followed by staining the strips specifically for enolase activity. These activities are referred to as enolases 1 through 5, with enolase 1 exhibiting the highest mobility toward the anode. Enolases 1, 4, and 5 were found to be very unstable and disappeared completely during storage or during the purification procedures. The two relatively stable forms (enolases 2 and 3) were purified and partially characterized. Both isoenzymes are composed of subunits of approximately 50,000 daltons and both isoenzymes behaved as dimers during centrifugation in sucrose gradients under nondenaturing conditions. The two enzymes were distinguished on the basis of their sensitivities to heat denaturation but not on the basis of electrophoretic analysis in the presence of 8 M urea nor by the size distribution of peptides generated by treating the enzymes with Staphylococcus aureus V8 protease. Treatment of enolase 2 with a number of diverse agents in vitro (H+, OH-, urea, guanidine hydrochloride, and iodide ions) resulted in the conversion of this isoenzyme to a species similar to enolase 3 on the basis of electrophoretic mobility and sensitivity to heat denaturation. Our observations are consistent with the view that enolases 2 and 3 may represent two different thermodynamically metastable conformations of the same protein species. Within this view, the thermodynamically least stable form (enolase 2) would be the most enzymatically active one and the one most resistant to heat denaturation. Several scenarios are given which may explain how multiple, thermodynamically metastable conformations of ascarid enolases could be generated in vivo.

The existence of multiple molecular forms of a large number of enzymes is now well established and much attention has been focused on elucidating the biochemical basis for this molecular multiplicity. Some isoenzymes, such as the well characterized multiple forms of lactate dehydrogenase (1), aldolase (2), and creatine-P kinase (3), are produced as a result of genetic multiplicity via the association of homologous, yet distinct subunit types into oligomeric combinations. Multiple forms of enzymes may also be produced by "epigenetic" processes (4) and, as such, these forms may more appropriately be termed "pseudoenzymes" to distinguish them from isoenzymes which have an underlying genetic basis. Pseudoenzymes may be produced by a number of postsynthetic processes, including molecular aggregation (5), derivative formation (6, 7), proteolytic modification (8), or other phenomena (9) which act on a single gene product. A final class of pseudoenzymes have been referred to as "conformational" variants and these forms are believed to arise as a consequence of a single molecular species assuming multiple, metastable conformational states.

The existence of conformational isoenzymes of a number of enzymes has been reported. However, a clear demonstration that multiple forms of an enzyme differ in conformation only rather than in subtle structural attributes is often difficult to make (10). For example, in the case of the multiple forms of "soluble" aspartate transaminase, Martinez-Carrion et al. (11) have reported that the isoenzymes could not be distinguished on the basis of a number of structural criteria, including amino acid composition, tryptic peptide analysis, Ouchterlony double diffusion tests, and molecular weight. However, they also found that interconversion of the multiple forms did not occur following the unfolding of the native enzymes; interconversion of these forms would have been expected if the isoenzymes differed in conformation only since, in the unfolded state, a single protein species should not have the capacity to "remember" a particular conformation it processed previously. In the case of mitochondrial malate dehydrogenase, Kitto et al. (12) presented electrophoretic evidence which suggested that some forms of the enzyme could be converted into others following denaturation of the molecules but later reported that the native and renatured isoenzymes differed in thermal stabilities (13). Furthermore, Mann and Vestling (14) presented evidence that the multiple isoenzymes are generated by the combination of structurally nonidentical subunits into oligomeric combinations.

Rothstein and associates (15, 16) have recently shown that enolases isolated from "young" and "old" free living nematodes (Turbatrix aceti) are chemically equivalent molecules but differ in conformation. These conformers were distinguished on the basis of catalytic activity and heat sensitivities but could not be separated on the basis of charge. They further showed that following reversible denaturation of these molecules, both enzymes assumed a very similar, if not identical, conformation and that this conformation was similar to that possessed by the native old enolase (16). In the present work, we give evidence for the existence of electrophoretically distinct conformers of enolase in the parasitic round worm Ascaris suum. Two relatively stable forms of ascarid enolase have been partially purified and characterized. Following treatment of these enzymes with a variety of agents, one isoenzyme is converted to a species similar to the other one as judged by electrophoretic behavior and heat sensitivities of native and treated enzymes. Our results are discussed in terms...
of the mechanisms by which multiple, metastable enolase conformers may be generated in vitro.

EXPERIMENTAL PROCEDURES

Materials—Live A. suum were obtained from commercial hog butchers and were maintained in 0.9% NaCl at 37°C overnight before use. Under these conditions, the worms remained viable for several days. Enzymes used as molecular weight markers, chicken aldolase, chicken creatine-P kinase, and rabbit enolase were isolated from skeletal muscle by the procedures recently described by us (17). The chicken creatine-P kinase, and rabbit enolase were isolated from 12,000 x g insoluble fraction of muscle homogenates was used as a source of actin subunits and bovine serum albumin was obtained from Sigma. Purified V8 protease was purchased from Miles. All other enzymes and substrates were obtained from Sigma.

Enzymatic Activity Measurements and Protein Determinations—Protein concentrations of crude and purified enolase preparations were determined by a modification of the procedure described by Lowry (18). Enolase activity was determined by a modification of the spectrophotometric assay described by Wold (19). Enolase activity is expressed as enzyme units per mg of protein.

Electrophoretic Methods—Cellulose polyacetate electrophoresis of crude and purified enolase preparations was performed at 250 volts for 2 h (20). The buffer contained 5 mM MgSO₄ and 1 mM EDTA. The strips were stained for enolase activity by laying the strips on a solution of NADP⁺ (monosodium salt); 100 μl of 0.66 M glucose; 100 μl of 0.1 M ADP; 40 μl of glucose-6-P dehydrogenase (20 units/ml); 5 μl of hexokinase (6600 units/ml); 5 μl of pyruvate kinase (380 units/ml); 50 μl of 2-P-glycerate (sodium salt); 1 ml of nitro blue tetrazolium (10 mg/ml); and 0.6 μl of phenazine methosulfate (1 mg/ml). Gels, about 4 mm thick, were poured in Petri dishes and were stored at 4°C until use. “Control” gels were prepared as described above except that 2-phosphoglycerate was omitted.

Electrophoresis in 5% or 15% polyacrylamide slab gels which contained 0.1% sodium dodecyl sulfate was performed using the gel reagents and buffers suggested by Laemmli (21). Electrophoresis in 5% polyacrylamide slab gels was performed as previously described (22) except that the gel solution was prepared in the presence of 8 M urea (Schwarz/Mann ultrapure) and samples were incubated in 8 M urea for 30 min prior to analysis. All gels were stained for protein with Coomassie blue.

Preparation of Tissue Fluids and Extracts—“Tissue fluids” of ascarid body wall and internal organ fractions were prepared by mincing the tissues with scissors and collecting the resulting liquid material in a glass capillary. Tissue extracts were prepared by homogenization of body walls or internal organs in 10 volumes of 10 mM Tris-HCl, 100 mM MgSO₄, 1 mM EDTA, pH 7.5, for 3 min in the Sorvall Omni-Mixer maintained in an ice bath. Soluble protein was obtained by centrifugation of the extracts at 12,000 x g for 20 min.

Purification of Enolases 2 and 3—The two stable enolase activities detected in extracts of ascarid body walls were purified as follows. The soluble protein fraction obtained from 90 g of body walls was adjusted to 35% saturation by the addition of solid ammonium sulfate. After stirring for 30 min in the cold, the precipitated fraction was collected by centrifugation and was discarded. The 35% ammonium sulfate supernatant fraction was adjusted to 55% saturation by addition of solid salt and, after stirring for 30 min, the precipitated proteins were collected by centrifugation. The precipitate was dissolved in a small amount of homogenization buffer. After extensive dialysis, the preparation was centrifuged and the supernatant fraction was applied to a DEAE-cellulose column (4.5 x 19 cm) (Schleicher/Schuell) which was equilibrated in the same buffer. Material not retained on the column, including the enolases, was applied to a DEAE-cellulose column (2.5 x 70 cm) (Whatman) which was equilibrated in 10 mM Tris-HCl, 5 mM MgSO₄, and 1 mM EDTA, pH 7.5. The column was washed with buffer until the A₂₈₀ of the effluent was less than 0.01. Then, the enolases were eluted from the column with a 0 to 0.5 M NaCl gradient which was prepared in the same buffer (1200 ml total). Peak fractions containing enolases 2 and 3 were pooled separately and these preparations were concentrated to about 0.5 mg of protein/ml by ultrafiltration. The enolase preparations were stored at 4°C or frozen at −20°C. See appropriate figure and table legends for any additional procedures.

RESULTS

Detection and Isolation of Ascarid Enolase Isoenzymes—Multiple enolase activities were detected when tissue fluids of body wall and internal organ fractions of ascarid worms were subjected to electrophoresis on cellulose polyacetate strips followed by staining the strips specifically for enolase activity (Fig. 1, left). If the enolase substrate 2-P-glycerate was omitted from the staining solution, none of these five activities were detected. The five enolase activities have been named according to their migration toward the anode, with enolase 1 being the most anodic species. When tissue extracts, rather than tissue fluids, were analyzed, different electrophoretic patterns were apparent (Fig. 1, middle and right). As shown, extracts of body walls contained predominantly enolases 2 and 3, while extracts of internal organs contained predominantly enolase 3. Similar electrophoretic profiles were obtained when extracts of the body wall fraction were prepared from pooled or individual worms, from fresh or frozen worms, and from young (small, sexually immature) or old (large, sexually mature) worms. Furthermore, no appreciable differences in electrophoretic profiles were observed when extracts were prepared in the absence or presence of 0.1 M 2-mercaptetoanhydride (to reduce disulfide bonds) or in the absence or presence of 0.1 mM phenylmethylsulfonyl fluoride and 3 mM o-phenanthroline (to inhibit proteolysis).

No attempt was made to isolate enolases 1, 4, and 5 since these three activities were found to be very unstable and disappeared completely upon storage or during extract fractionation. The two relatively stable isoenzymes (enolases 2 and 3) were purified from body walls by ammonium sulfate fractionation and ion exchange chromatography as described under “Experimental Procedures.” As shown in Fig. 2, the two isoenzymes could be separated from each other by DEAE-cellulose chromatography. The purities of the two enolase preparations were ascertained by electrophoretic analysis in 9% polyacrylamide gels containing sodium dodecyl sulfate. Densitometric analysis of the stained gels revealed that the enolase 2 and 3 preparations were greater than 60% pure. (Data not shown.)

Molecular Properties of Enolases 2 and 3—Several structural properties of enolases 2 and 3 were compared in the middle and right panels. Enolase activities present in fluids of body walls (A) and internal organs (B). Middle and right panels, enolase activities present in extracts of body wall and internal organ fractions, respectively. The faint activity located between enolases 4 and 5 was developed in the absence of substrate and, therefore, is not a bona fide enolase activity.

Fig. 1. Electrophoretic resolution of multiple ascarid enolases. Fluids and extracts of body wall and internal organ fractions of worms were prepared as described under “Experimental Procedures.” Samples were subjected to cellulose polyacetate strip electrophoresis and the strips were stained for enolase activity. Left panel, enolase activities present in fluids of body walls (A) and internal organs (B). Middle and right panels, enolase activities present in extracts of body wall and internal organ fractions, respectively. The faint activity located between enolases 4 and 5 was developed in the absence of substrate and, therefore, is not a bona fide enolase activity.
matography on DEAE-cellulose. The enolases present in extracts of body walls were partially purified and then were separated from these multiple enzymes. The subunit molecular weights of the enzymes are not responsible for the observed multiplicity. Our hope of determining the biochemical basis for the existence of dimers. Thus, differences in aggregation states of these enproteins of known molecular weights as standards.

Two isoenzymes were determined by electrophoresis in 9% polyacrylamide gels containing sodium dodecyl sulfate with electrophoresis in polyacrylamide gels containing 8% urea. The enzyme subunits displayed identical mobilities during electrophoresis in polyacrylamide gels containing 8% urea (data not shown). Furthermore, as shown in Fig. 5, the two enzymes generated identical peptide patterns when subjected to limited proteolysis by S. aureus V8 protease followed by resolution of the peptides by electrophoresis in 15% polyacrylamide gels in the presence of sodium dodecyl sulfate.

Catalytic Properties of Enolases 2 and 3—Since the two enzyme preparations were not homogeneous, we could not measure the specific catalytic activities of the ascarid enolases directly. Instead, we measured the specific activities of the preparations and corrected these values for the degrees of contamination with other proteins by electrophoretic and densitometric analysis of the purified preparations. We are assuming that there is no preferential binding of Coomassie blue to these proteins. The specific catalytic activity calcu-

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**Fig. 2.** Separation of enolases 2 and 3 by ion exchange chromatography on DEAE-cellulose. The enolases present in extracts of body walls were partially purified and then were separated from each other by DEAE-cellulose chromatography as described under "Experimental Procedures." Fractions were pooled as indicated by the brackets. Comparisons between the isoenzymes present in the initial sample (S) and those in the enolase 3 and 2 preparations were made by cellulose polyacetate strip electrophoresis (see inset).

**Fig. 3.** Subunit molecular weights of ascarid enolases 2 and 3. Ascariid and rabbit muscle enolases as well as proteins of known molecular weight were electrophoresed in adjacent wells of the same 9% polyacrylamide slab gel. Standard proteins were bovine serum albumin (bsa) (Mr = 68,000) (33), chicken actin (act) (Mr = 48,000) (26), chicken muscle creatine phosphokinase (cpk) (Mr = 43,000) (34), and chicken muscle fructose diphosphate aldolase (ald) (Mr = 40,000) (35). The molecular weights of the standard proteins were plotted versus their migration into the gel relative to the migration of the bromphenol blue tracking dye. Molecular weights of the enolase subunits were calculated from this standard curve. Note that all enolase subunits were found to have the same subunit molecular weights (Mr = 50,000-51,000).

**Fig. 4.** Subunit structure of ascarid enolases 2 and 3. Samples (0.5 ml each) of enolases 2 and 3 containing 2 to 3 units of activity were applied to separate 5%--20% sucrose gradients (13 ml each) prepared in 100 mM Tris-HCl, pH 7.5. The gradients were centrifuged at 39,000 rpm for 24 h in the Beckman SW 41 rotor. Then 8-drop fractions of the gradients were collected by pumping the contents of each tube from the bottom to the top with 55% sucrose. Each fraction was assayed for enolase activity. The position of fructose diphosphate aldolase (Mr = 160,000) in similar gradients is included for comparison.
reported for the human (27) and salmon (19) muscle enzymes. The activity of enolase 2 (60-70 units/mg) was comparable to those calculated for enolase 3 (30-40 units/mg). Limited proteolysis of the enolases by S. aureus V8 protease was carried out for 30 min at 37 °C by the method recently described by others (36, 37). Then, the peptides generated from the enolases were resolved according to size by electrophoresis and their positions in the 15% gel were visualized by Coomassie blue staining. None of the indicated peptides were visualized when V8 protease was analyzed separately.

As shown in Fig. 6, enolase 3 was considerably more sensitive to heat inactivation than was enolase 2. The inactivation of enolase 3 at 55 °C was found to follow first order kinetics and the partial inactivation of enolase 2 at 55 °C reflected the presence of a small amount of enolase 3 in this particular preparation as judged by electrophoretic analysis of enzyme preparations before and after heating (Fig. 7). We ruled out the possibility that differences in amounts of stabilizing or destabilizing components in the two preparations were responsible for the different heat stabilities of the enolases by demonstrating that the shape of the heat inactivation curve of a sample containing a mixture of the two isoenzymes was intermediate between those generated by enolases 2 and 3 separately.

Conversion of Enolase 2 to a Species Similar to Enolase 3 in Vitro—We routinely observed some conversion of enolase 2 to a species which expressed the same electrophoretic mobility as enolase 3 during prolonged storage of the preparations at 4 °C or by repeated freezing and thawing of enolase 2 preparations. In this section, we demonstrate that enolase 2 can be converted to a species similar to enolase 3 by a number of agents, as judged by the electrophoretic behavior and heat sensitivities of native and treated enzymes. As shown by the results presented in Fig. 8, no change in the electrophoretic mobility of enolase 3 was observed after treating this isoenzyme at reduced pH. In contrast, after incubation of enolase 2 in acidic solutions (pH 2 to 5), this isoenzyme was converted to an electrophoretic species indistinguishable from enolase 3. This isoenzyme conversion was also suggested by comparing the heat sensitivities of the treated enzymes. As shown in Fig. 9, treated enolases 2 and 3 displayed similar sensitivities to heat inactivation and these sensitivities were similar to that expressed by native enolase 3. (See Fig. 6 for comparison.)

The existence of a minor heat-stable component in the treated enolase 2 preparation presumably reflects the nonquantitative conversion of enolase 2 as judged by electrophoretic analysis of the heated enzyme preparation (data not shown).

The isoenzyme conversion was not caused by an acid-catalyzed covalent change in the structure of enolase 2 since the conversion was also observed by treating the enzyme with base (pH 12.5), 8 M urea, 1.5 M guanidine hydrochloride, or 1.6 M KI (see below). However, no isoenzyme conversion was observed after treating the enzymes with 100 mM 2-mercaptoethanol. The recovery of activity after treatment of enolase 2 (about 60%) was always less than that of enolase 3 (about 80%). This difference was expected since enolase 2 appears to be somewhat more enzymatically active than enolase 3.

Neutralization of the acid-treated enzymes in the presence of low (1 mM) or high (100 mM) concentrations of magnesium or in the presence or absence of substrate (1 mM 2-P-glycerate) did not influence the isoenzyme conversion. Also, no effect on the electrophoretic mobilities of the isoenzymes was observed by treatment with S. aureus V8 protease. Enolase preparations were subjected to electrophoresis in a 9% polyacrylamide slab gel in the presence of sodium dodecyl sulfate. The gel was stained for protein and the regions of the gel containing enolase subunits were cut out and placed in sample wells of a 15% polyacrylamide slab gel containing 0.1% sodium dodecyl sulfate. Limited proteolysis of the enolases by S. aureus V8 protease was analyzed for 15 min at 37 °C by the method recently described by others (36, 37). Then, the peptides generated from the enolases were resolved according to size by electrophoresis and their positions in the 15% gel were visualized by Coomassie blue staining. None of the indicated peptides were visualized when V8 protease was analyzed separately.

As shown in Fig. 9, treated enolases 2 and 3 displayed similar sensitivities to heat inactivation and these sensitivities were similar to that expressed by native enolase 3. (See Fig. 6 for comparison.)

[Fig. 5. Size distribution of peptides generated from enolase 2, enolase 3, and enolases 2 plus 3 by treatment with S. aureus V8 protease. Enolase preparations were subjected to electrophoresis in a 9% polyacrylamide slab gel in the presence of sodium dodecyl sulfate. The gel was stained for protein and the regions of the gel containing enolase subunits were cut out and placed in sample wells of a 15% polyacrylamide slab gel containing 0.1% sodium dodecyl sulfate. Limited proteolysis of the enolases by S. aureus V8 protease was carried out for 30 min at 37 °C by the method recently described by others (36, 37). Then, the peptides generated from the enolases were resolved according to size by electrophoresis and their positions in the 15% gel were visualized by Coomassie blue staining. None of the indicated peptides were visualized when V8 protease was analyzed separately.]

[Fig. 6. Sensitivities of enolases 2 and 3 to inactivation by heat. Preparations of enolases 2 and 3 were extensively dialyzed against 10 mM Tris-HCl, 5 mM MgSO₄, and 1 mM EDTA, pH 7.5, and were then diluted to about 2 units/ml. Aliquots (300 µl each) were placed in 1.5-ml conical polypropylene tubes and were then incubated at elevated temperatures. Left panel, inactivation of enolase activity by heating for 10 min at the indicated temperatures. Right panel, time course of enzyme inactivation of enolase 2 and enolase 3 at 55 °C.]

[Fig. 7. Electrophoretic analysis of control and heated enolase preparations. Samples of enolases 2 and 3 were heated at 55 °C for 15 min, while control samples were stored at room temperature. Then aliquots of the preparations containing approximately equal amounts of activity were subjected to cellulose polyacetate strip electrophoresis. In each comparison, the heated sample appears on the right. The small amount of isoenzyme 3 present in the enolase 2 preparation was completely removed during incubation at 55 °C.]
after extensive dialysis against 50 mM EDTA nor after incubating the isoenzymes in the presence of crude tissue extracts. These observations suggest that differences in amount of bound ligands in enolases 2 and 3 were not responsible for the observed multiplicity.

The isoenzyme conversion appeared to be associated with some degree of dissociation of the enzymes. After treating samples containing mixtures of ascarid and rabbit enolases under the conditions which resulted in isoenzyme conversion, enzymatically active heterodimers containing one ascarid and one rabbit enolase subunit were detected by electrophoresis. (Fig. 10). In contrast, no isoenzyme conversion or heterodimer formation was observed when mixtures of these enzymes were incubated for the same length of time in buffer alone.

![Fig. 8. Apparent conversion of enolase 2 to enolase 3 by incubation at reduced pH.](image)

**DISCUSSION**

The present work demonstrates the existence of multiple electrophoretic forms of enolase in tissue fluids and extracts of the parasitic round worm *A. suum*. We became interested in determining the biochemical basis for the multiple ascarid enolases because only two of the electrophoretic forms of the enzyme were stable; since enolase is a dimeric molecule, a three-membered set of stable activities, not a two-membered one, would have been expected if the multiplicity was generated as a consequence of the combination of two chemically dissimilar subunits into dimers. No differences in the relative levels of enolases 2 and 3 were observed when tissue extracts or purified isoenzymes were incubated in the presence or absence of high concentrations of 2-mercaptoethanol which suggests that the multiplicity was not due to different oxidation states of cysteinyl residues of the enzyme. The presence or absence of phenylmethylsulfonyl fluoride and o-phenanthroline during isoenzyme purification did not affect the relative distribution of the two isoenzymes and both forms were found to be composed of subunits of indistinguishable molecular weight; thus, the multiplicity does not appear to result from proteolytic modification of a single molecular species. The multiplicity was not caused by differ-

![Fig. 9. Sensitivities of renatured enolases 2 and 3 to inactivation by heat.](image)
ences in aggregation states of enolase subunits since both isoenzymes behaved as dimers during centrifugation in sucrose gradients. Finally, no differences in primary structure of the isoenzymes were indicated by electrophoretic analysis in the presence of 8 M urea nor by the size distribution of peptides generated by treating the enolases with S. aureus V8 protease.

The observations that enolase 2 could be converted to a species which exhibited similar electrophoretic mobility and thermal stability as did enolase 3 give further support to the premise that the two isoenzymes are structurally very similar. The argument that the isoenzyme conversion was caused by the breakage of covalent bonds such as would occur during dephosphorylation or deamidation seems highly unlikely. The agents which caused the conversion (H+, OH−, urea, guanidine hydrochloride, and KI) would not all participate in the same chemical reaction and the relatively mild treatments which effected the conversion should not result in the breakage of covalent bonds of any kind. Thus, it is likely that enolases 2 and 3 have equivalent covalent structures.

Differences in the amounts of bound ligands present in identified proteins may cause the appearance of enzyme multiplicity, and this mechanism appears to be responsible for the generation of some multiple forms of alcohol dehydrogenase in Drosophila (9). However, we found no change in the electrophoretic mobilities of enolases 2 and 3 after incubation of the enzymes in tissue fluids. Magnesium ions have been shown to have complex effects on the structure of enolase (28-30), but no effect on the electrophoretic mobility of enolases 2 and 3 was observed after dialysis of the isoenzymes against 50 mM EDTA for 1 day. Also, the unidirectional conversion of enolase 2 to 3 was the same whether renaturation of unfolded enolase subunits was performed in the presence of low (1 mM) or high (100 mM) concentrations of magnesium, and the presence or absence of substrate in the renaturation medium had no effect on the isoenzyme conversion. These observations suggest that differences in content of noncovalently bound ligands in enolases 2 and 3 were not responsible for the enzyme multiplicity.

Taken together, the present observations would be consistent with the view that enolases 2 and 3 may represent two different metastable active conformations of the same protein species. Within this view, enolase 2 would be the more enzymatically active conformer and the one more resistant to heat denaturation as suggested by the catalytic properties of the two isoenzymes. These observations imply that the activation energy barrier for the conversion of conformer 2 to an enzymatically active (denatured) form is greater than that for the denaturation of enolase 3. Some dissociation of enolase dimers was found to occur under the conditions which resulted in the isoenzyme conversion. Consequently, within the present context, it may be possible that some relaxation of subunit-subunit interactions within the enolase dimer is necessary to "unlock" the molecule from conformation 2 so that it can assume a thermodynamically more stable conformation. This thermodynamically stable conformation appears to be similar to that possessed by "native" enolase 3.

The possibility that the ascarid enolases are conformational isoenzymes is of interest in view of the recent observations of Rothstein and associates (15, 16) on the properties of enolase molecules isolated from young and old free-living nematodes. They gave firm evidence that enolases isolated from young and old T. aceti are chemically equivalent molecules and differ in conformation only. They also gave evidence that a unidirectional conversion of the young conformer to a species similar to the native old one could be effected in vitro and that the young conformer was more enzymatically active and more resistant to heat denaturation than was the old conformer (15, 16). Thus, ascarid enolases 2 and 3 may be analogous to the young and old conformers of the T. aceti enzyme, respectively. However, in the case of the T. aceti enzymes (15), no differences in charge of young and old enzyme were observed, while the ascarid enolases were readily resolvable by electrophoresis and ion exchange chromatography. Also, the production of the old conformer in T. aceti appears to be caused by age-related processes and may reflect a longer "dwell time" of enolase molecules in old organisms as compared with young ones (15, 16, 31). In contrast, the existence of ascard enolases 2 and 3 does not appear to be associated with age-related processes since we found no appreciable differences in the distribution of these isoenzymes in small, sexually immature and large, sexually mature worms. It should also be mentioned that Porcelli et al. (32) recently gave preliminary evidence for the existence of minor species of yeast enolase which may represent conformational variants of the major form of the enzyme.

The present work raises the question of how multiple, enzymatically active enolase conformers could be generated in vivo. Unique environmental conditions existing in intact cells or intracellular compartments may allow enolase molecules to assume conformations which would not be tolerated during the folding of enolase polypeptides in vitro. Alternatively, enzymatically active, thermodynamically unstable conformations of the enolase molecule may be the only ones produced in vivo. It is not too difficult to envision that the initial folding of nascent polypeptide chains during the translation step of protein synthesis could create a "nucleation site" which would prevent subsequently added amino acid residues from participating as fully in conformational decisions as they would do during the refolding of complete enolase polypeptide chains in vitro. In this view, the thermodynamically more stable conformers would be spontaneously formed by conformational rearrangements of the native conformers. The preferential removal of these non-native conformers by the degradative apparatus of the cell could allow for the existence of steady state levels of different conformers in vivo.

We have not observed any indication of the existence of electrophoretically distinct enolase conformers in a number of vertebrate tissues, nor in Drosophila (larva, adult) or wheat germ. Consequently, the enolase isoenzymes described here may have a restricted phylogenetic distribution. Nonetheless, these isoenzymes may be useful probes in elucidating the patterns of folding available to polypeptide chains in vitro and in vivo.

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