Studies on Yeast Enolase

QUANTITATIVE END GROUP ANALYSES AND THE EFFECT OF EXOPEPTIDASE DIGESTION*

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SUMMARY

The structural model of yeast enolase has undergone substantial modification in recent years, and the quantitative end group analysis of the enzyme was undertaken as a means of establishing the most recent two-subunit model by a chemical method. Carboxypeptidase digestion and hydrolysis gave 1.95 and 1.85 moles, respectively, of carboxyl-terminal terminal alanine per mole of enzyme, and amino-terminal analysis by the cyanate method gave 1.8 moles of amino-terminal alanine per mole of enzyme, thus confirming the model of yeast enolase as an 88,000-dalton protein consisting of two 44,000-dalton subunits. Contrary to reports in the literature, pure enolase was found to be very resistant to exopeptidase digestion. The earlier findings that large segments of both the amino-terminal and carboxyl-terminal sequences can be removed by exopeptidases without loss of enzyme activity can thus not be reproduced in this laboratory. Some possible reasons for this discrepancy are discussed.

Enolase (2-phospho-D-glycerate hydro-lyase; EC 4.2.1.11) from yeast was long thought to consist of a single polypeptide chain with a molecular weight of 67,000. The experimental bases for this model, the early molecular weight determinations, the quantitative amino-terminal analysis, and the early unsuccessful attempts to demonstrate dissociation into subunits have been reviewed (1). More recently, the concept that yeast enolase was a single chain protein was proven incorrect by unequivocal demonstrations that the enzyme can be dissociated into subunits with molecular weight equal to one-half that of the native enzyme (2, 3). This modified model of the enzyme as a dimer of 67,000 daltons made up of two subunits of 34,000 daltons was difficult to reconcile with the reported value of 0.85 to 0.95 moles of amino-terminal alanine per 67,000 g of enzyme (4), and the work reported here was undertaken to reexamine the chemical bases of the subunit structure through quantitative carboxyl- and amino-terminal analyses. While this work was in progress, an amino-terminal analysis of 2 moles of alanine per 67,000 g of protein was reported, providing chemical confirmation of the dimer model (5).

In our hands, the end group analyses persistently and reproducibly gave low results (1.4 to 1.8 moles/67,000 g of enzyme) (6), and this eventually led to a reinvestigation of the molecular weight of both the native enzyme and its subunits (7). As a result of all these studies, the current model of yeast enolase is a protein of 88,000 daltons containing two subunits of 44,000 daltons, and each subunit, as the data in this paper show, contains one amino-terminal alanine and one carboxyl-terminal leucine.

In the studies of the terminal residues of yeast enolase by the use of exopeptidases, we found that the enzyme is quite resistant to exopeptidase attack. This finding is in complete conflict with earlier reports that more than 20% of the total amino acid content of enolase can be removed from either the amino or the carboxyl end by leucine amino peptidase and by carboxypeptidase, respectively (8, 9). Since the question of just how much of the primary sequence of an enzyme is essential for activity is of obvious relevance to the formulation of structure-function models of enzymes, the exopeptidase action on enolase was studied in some detail. This paper thus reports the results of two separate but experimentally closely related studies: the quantitative end group analysis as an essential part of the evidence for the two-subunit structural model, and the reinvestigation of the exopeptidase digestion as an attempt to establish the involvement of the amino- and carboxyl-terminal sequences in the functional properties of yeast enolase.

EXPERIMENTAL METHODS

Materials

Enolase was prepared from several lots of fresh bakers' yeast (Fleischmann Company, Pekin, Illinois; the gift of Standard Brands, Inc., Minneapolis, Minnesota) by the method of Westhead and McLain (10) as reported by Westhead (11). The existence of multiple active forms of enolase from yeast is well established (10), and only the major component, enolase A, which is also the component that elutes first from TEAA-cellulose was used in this work (10). This form contained over 95% of a single electrophoretic species and had a specific activity of 650 ± 30 units per mg (reported value for the purest prepa-
Carboxypeptidase A (Lots 7FA, 9BA), carboxypeptidase B (Lots 7AA, 9DA), leucine aminopeptidase (Lot SJA), all diisopropyl fluorophosphate-treated, and twice recrystallized lysozyme were obtained from Worthington. Aminopeptidase M, the product of Rohm and Haas, Darmstadt, Germany, was obtained from Henley and Company, New York. Bovine insulin was obtained from Mann and was oxidized with performic acid by the method of Hirs (12). Bovine pancreatic ribonuclease (Sigma, five times crystallized, type 1A) was purified chromatographically according to Hirs, Moore, and Stein (13) and was the gift of Mr. C. Chin. The substrate, barium n-glycerate 2-phosphate (Sigma) was converted to and used as the water-soluble cyclohexylammonium salt. All other chemicals were of the gift of Mr. C. Chin. The substrate, barium n-glycerate 2-phosphate (Sigma) was converted to and used as the water-soluble cyclohexylammonium salt. All other chemicals were commercial samples of highest available purity. A commercial source of high purity ion-free water was used throughout.

Methods

Enolase Activity Assay—These assays were performed by measuring initial rates of increase in absorbance at 230 nm due to production of phosphoenolpyruvate at 30° (11). The standard assay medium contained 2 mM n-glycerate 2-phosphate, 1 mM magnesium acetate, and 0.01 mM EDTA in pH 7.8 Tris-acetate buffer (50 mM in acetate).

Protein Assays—These assays were based on the ultraviolet absorption with the use of published values for the absorbance. The value A₂₈₀ untreated sample was 0.8955 for enolase (1).

Automatic Amino Acid Analyses These analyses were performed on a Beckman-Spinco 120C amino acid analyzer. The ninhydrin peaks were integrated with the use of a Beckman-Spinco 125 integrator.

Carboxy- and Amino-terminal Analyses

Carbamylation—Carbamylaminobutyric acid and carbamylalanine were first prepared to be used as internal standard and control. dL-α-Aminobutyric acid (K and K Laboratories, Plainview, New York) was carbamylated by warming with excess KNCO (14), and, after acidification, the reaction mixture was evaporated to dryness. The carbamylamine acid was extracted into ethanol and, after evaporation of the ethanol, was dissolved in water and subjected to chromatography on Dowex 50 (H⁺). Amino acid analysis by the long column procedure for neutral and acidic amino acids showed the carbamylaminobutyric acid to contain less than 1% of ninhydrin-positive material. L-Alanyl-L-valine (Mann) was carbamylated, isolated, and analyzed in the same way. The concentrations of stock solutions of the carbamylamino acid or peptide were determined by quantitation of the regenerated amino acids after hydrolysis of duplicate samples with 6 N HCl at 110° for 100 hours. The peptide concentration was calculated from recoveries of valine (which were 7% higher than those of alanine).

A 50-mg sample of enolase was carbamylated according to Stark (15), and a 25-mg blank sample was carried through in parallel, but in the absence of urea and KNCO. Aliquots of both samples were removed for acid hydrolysis in order to determine protein concentration by amino acid analysis. The recovery in the subsequent steps was evaluated by the use of both internal and external standards as follows. A measured amount of carbamylaminobutyric acid was added as an internal standard to the enolase samples, and, in addition, separate duplicate samples of carbamylaminobutyric acid and carbamylalanine were carried through the same procedures in parallel with the enolase samples.

Preparation of Enolase for Exopeptidase Digestion and for Hydrazinolysis—Enolase solutions were prepared as follows, acid-washed glassware being used throughout. Lyophilized enolase A was dissolved by slow addition to a small amount of ice-cold water under continuous stirring in an ice bath to yield a concentration of 10 to 20 mg per ml. Any insoluble protein was removed by centrifugation, and the clear supernatant was passed through a column (1.3 × 22 cm) of mixed-bed ion exchange resin (Rexyn 300, Fisher) to eliminate any free amino acids. Small fractions (approximately 1 ml) were collected, their absorbance at 280 nm was determined, and all fractions with protein concentration greater than 5 mg per ml were pooled. Appropriate dilutions were made with a Tris-Mg⁺⁺ buffer (double strength assay buffer) and the final protein concentration was determined from the A₂₈₀ reading. Samples were also taken for acid hydrolysis in order to check protein concentration by amino acid analysis. Molar concentrations were calculated with the use of 88,000 as the molecular weight (7). Each purified enolase sample contained the appropriate amount of norleucine or β-alanine or both which served as internal standards for the amino acid determinations. Norleucine at a level of 1 to 2 μmole per μmole of enolase was used to standardize the small quantities of amino acids released in the end group determinations while β-alanine at a level of 100 μmole per μmole of enolase was used to standardize the larger quantities of amino acids in the acid-hydrolyzed samples.

Hydrazinolysis—The procedure of Braun and Schroeder (16) was used, except that the hydrazine (City Corporation, New York, 95%+) was used without further purification. Protein solutions (enolase and egg white lysozyme as a control) containing norleucine at a known concentration (from 1 to 2 μmole per μmole of protein) were pipetted into 5-ml Pyrex hydrolysis vials. The samples, containing from 0.1 to 0.25 μmole of protein, were frozen and lyophilized individually. In the case of enolase which had been exhaustively digested with carboxypeptidase A, the protein was precipitated with trichloroacetic acid and washed in sequence with 95% ethanol, absolute ethanol, acetone, and ether. After drying under vacuum, from 4 to 8 mg of the protein were weighed to the nearest 0.01 mg into Pyrex vials. Amberlite CG-50 resin (50 mg) was added to the protein in each vial, and the samples were dried overnight at 65° under vacuum in an Aberdeen drier containing P₂O₅. After the addition of hydrazine and sealing the frozen samples under vacuum, the mixtures were heated at 50° or 85° for the desired length of time.

In order to analyze for neutral and acidic amino acids, the hydrazinolysate was lyophilized, extracted, and chromatographed on Amberlite CG-50 resin according to Braun and Schroeder (16). The amounts of amino acids recovered were corrected to 100% recovery of the added norleucine standard.

The analysis for basic amino acids was a modification of the methods of Winstead and Wold (17) and Hartman and Wold (18); the long column of the amino acid analyzer was equilibrated and eluted with pH 5.28 buffer, followed by pH 9.7 borate buffer after 35 min of operation.

Exopeptidase Digestions—Concentrated buffer solution was
added to measured amounts of enolase solution containing nor-
leucine as internal standard to bring the buffer concentration to approximately 0.05 M (the different buffers used are given with the results). A zero time sample was withdrawn for analysis for free amino acids. After temperature equilibration, an aliquot of exopeptidase stock solution was added to the experimental tubes and also to control tubes, containing only buffer. Samples from all tubes were removed at fixed times during the digestion. Samples for amino acid analysis were brought to 5% in trichloroacetic acid and centrifuged. The supernatant was lyophilized, and the residue was dissolved in water and relyophilized until residual trichloroacetic acid was removed. No free amino acids were ever detected in the undigested enolase. Amino acids released due to exopeptidase self-digestion (generally small in amount) were subtracted from the values obtained from enolase digestion. Samples for the digestion mixtures which were to be assayed for enolase activity were diluted directly into an appropriate volume of Tris-Mg++ buffer and assayed as described previously.

RESULTS AND DISCUSSION

In all quantitative analyses of macromolecular systems, the extensive use of controls and standards is particularly impor-
tant to keep a close check on manipulative and methodological sources of errors and to evaluate the quantitative recoveries from unknown systems relative to well characterized systems. In the work reported here, several known standards were used, and their selection was based on unique analytical advantages (norleucine, β-alanine, and aminobutyric acid have unique elution positions in the amino acid analysis) or on similarities to the unknown enolase, with the prior knowledge that enolase contains amino-terminal alanine and carboxyl-terminal leucine.

Quantitative End Group Analysis

Carboxypeptidase A Digestion of Enolase—The time course of amino acid release during digestion of enolase by carboxy-
peptidase A is shown in Fig. 1. A total of 1.95 moles of leucine were released per mole of enolase, release being complete in less than 1 hour. No other amino acids were detected. Digestion in the presence of 1 M KCl, which favors dissociation of enolase (2), gave identical results. Other variables, such as temperature, further addition of carboxypeptidase A (inset, Fig. 1), pH, buffers, and presence of urea were also tested but did not result in any increase in the leucine yield, nor did they lead to a release of amino acids other than leucine.

Hydrazinolysis—The release of carboxyl-terminal leucine from enolase and from the lysozyme control sample (carboxyl-
terminal Arg-Leu) as a function of time is shown in Table I. Although treatment for 46 hours at 85° was sufficient to release quantitatively the carboxyl-terminal leucine from lysozyme, prolonged treatment at 85° was required to approach a limiting value for enolase. A value of 1.86 moles of leucine per 88,000 g of enolase was obtained for the longest hydrazinolysis period. Phillips (19) has pointed out that, as the temperature is further increased in these experiments, free amino acids are converted to hydrazides, and it was not deemed desirable to attempt more severe conditions than those given in Table I. As expected, even under the conditions used, some side reactions are evident. Thus, trace quantities of alanine, serine, and aspartic acid and somewhat larger quantities of glycine (5 to 6% of the leucine values for lysozyme, and up to 20% of the leucine value for enolase) were found for both proteins.

Hydrazinolysis of Carboxypeptidase A-digested Enolase—In an attempt to identify the penultimate carboxyl-terminal residue in enolase exhaustively, carboxypeptidase A-digested enolase was subjected to hydrazinolysis. No acidic, neutral, or basic amino acids were released in this experiment. Thus, the carboxypeptidase A release of carboxyl-terminal leucine was shown to be complete, but the penultimate residue was not identified. Since arginine is destroyed by hydrazinolysis, and since asparagine and glutamine are lost as the monohydrazides in the procedures used, either of these 3 could be the penulti-
mate residue in enolase. We tend to rule out arginine as a possibility on two counts. First, if arginine were the penulti-
mate amino acid, hydrazinolysis should result in a rapid release of carboxyl-terminal leucine from enolase as was found for lysozyme. Second, if arginine were the penultimate residue, trypsin digestion should release free leucine. When this was checked under conditions in which trypsin was found to release

![Fig. 1. Amino acid release during carboxypeptidase A digestion of enolase. Enolase at 4.3 mg per ml was digested with diso-
propyl fluorophosphosphate-treated carboxypeptidase A at 30° in 0.10 M NH₄HCO₃ buffer, at a 1:20 molar ratio of carboxypeptidase to
enolase (▲). A parallel digestion in 0.05 M NH₄HCO₃ at 30° was increased to 37°, and a second addition of carboxypeptidase A at 30° in 0.10
M NH₄HCO₃ (X). Inset, enolase at 7.25 mg per ml was digested in NH₄HCO₃ at a 1:16 molar ratio of carboxypeptidase to
enolase. The digestion temperature of 30° was increased to 37° at 45 min. At 90 min a second addition of carboxypeptidase at a 1:11 molar ratio was made.

Table I

<table>
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<th>Protein</th>
<th>80°</th>
<th>85°</th>
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<tr>
<td></td>
<td>moles/mole protein</td>
<td>moles/mole protein</td>
</tr>
<tr>
<td>Lysozyme</td>
<td></td>
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<tr>
<td>hps</td>
<td>0.910 ± 0.024</td>
<td>0.977 ± 0.033</td>
</tr>
<tr>
<td>40</td>
<td>0.977 ± 0.023</td>
<td>0.977 ± 0.033</td>
</tr>
<tr>
<td>Yeast enolase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>1.38a</td>
<td>1.61a</td>
</tr>
<tr>
<td>120</td>
<td>1.80b</td>
<td>1.61b</td>
</tr>
<tr>
<td>95</td>
<td>1.51 ± 0.010</td>
<td>1.61 ± 0.05</td>
</tr>
<tr>
<td>120</td>
<td>1.80b</td>
<td>1.61b</td>
</tr>
</tbody>
</table>

a Single determinations.

b An unusually high glycine release (0.4 μmole) was observed in this experiment.
alanine from the Pro-Lys-Ala sequence of oxidized insulin (B chain), no leucine was released from enolase.

The carboxy-terminal analyses by both enzymatic and chemical methods are in good agreement at 1.9 ± 0.05 moles of leucine per 88,000 g of enzyme, and it can also be tentatively concluded from the data that the penultimate residue is either asparagine or glutamine.

Carbamylamylaminobutyric acid gave 93.2 ± 2.9% of the expected absolute recoveries. Small and variable amounts of glycine and alanine were also observed. (Stark (15) has reported that these amino acids result from the decomposition of hydantoins of several amino acids.) A sample of carbamylalanine yielded 91.8% of the expected NH-terminal alanine in our hands (on an absolute recovery basis). When normalized to the internal standard, the yield of alanine from carbamyl-enolase was 1.88 ± 0.06 moles of alanine per mole of enolase. When corrected for the background value of 0.03 mole of alanine per mole of enolase from the blank, this value is reduced to 1.85 moles of alanine per mole of enolase.

There are several sources of error in this procedure, and a variety of yields of amino-terminal amino acids has been reported. Fortunately, it is possible to check the efficiency of individual steps. Thus, the conversion of lysine to citrulline is a good marker for the efficiency of the carbamylamylaminobutyric acid reaction, and the finding that 75.5 ± 1.2% of the lysine persisted as citrulline after acid hydrolysis (6 N HCl, 110°C, 22 hours) in this work is a good indication that the reaction went to completion (15). The cyclization and hydrolysis steps are more problematic and can only be evaluated by the use of model compounds in parallel experiments. It has been suggested that the yields from the cyclization and the hydrolysis vary with the nature of the particular amino acids involved in the bond to be broken, and recovery of the amino-terminal amino acid from different proteins of known sequence varies considerably. Thus, the amino-terminal amino acid was obtained from insulin A chain (Gly-Ile-) in 95% yield, from the B chain (Phe-Val-) in 85% yield (14) whereas from the a chain of a hemoglobin with an amino-terminal Val-Leu- sequence, the yield was 63 to 70% (20), and from human transferrin (aminoterminal valine) the yield was only 50% (21). There is no evidence that suggests that the proposed Ala-Gly-amino-terminal sequence of enolase (4) should present any particular problems in this reaction, and the value of 1.85 moles of alanine per mole of enzyme taken together with the 91.6% yield of alanine from the carbamylvamylalanylvaline control in the parallel experiment should be considered a valid quantification of the amino terminus of enolase.

Digestion with Aminopeptidase—The rate of release of amino acids from enolase treated with aminopeptidase was investigated. It was confirmed that alanine was the amino acid released in greatest amount at early stages of digestion with aminopeptidase M, but the release of alanine was extremely slow and no useful quantification of amino-terminal alanine could be obtained by this method. These experiments are discussed below.

The results from all of these experiments give a consistent picture of the structure of enolase as consisting of two polypeptide chains of approximately 44,000 daltons, each with a carboxyl-terminal leucine and an amino-terminal alanine residue.

Effect of Exopeptidase Digestion on Enolase Activity—The report in the literature that up to 23% of the polypeptide chains of approximately 44,000 daltons, each with a carboxyl-terminal leucine and an amino-terminal alanine residue, could be obtained by this method. These experiments are discussed below.

The results from all of these experiments give a consistent picture of the structure of enolase as consisting of two polypeptide chains of approximately 44,000 daltons, each with a carboxyl-terminal leucine and an amino-terminal alanine residue.

Carboxypeptidase A digestion: enolase, 7.7 mg per ml in 0.06 M Tris chloride buffer containing 5.0 × 10⁻⁵ M Mg⁺⁺, pH 7.75, was digested with carboxypeptidase A at a 1:45 molar ratio of carboxypeptidase to enolase at 30°C. Aminopeptidase M digestion: enolase, 9.4 mg per ml in 0.06 M potassium phosphate buffer containing 6.0 × 10⁻⁵ M Mg⁺⁺, pH 6.9, was digested with 0.1 mg of aminopeptidase M per mg of enolase, at 30°C. In both cases, samples of 15 to 25 µl from both the digestion mixtures (△) and from the digestion-free controls (●) were removed at timed intervals and diluted with the Tris-Mg⁺⁺ assay buffer for activity determinations.

FIG. 2. The effect of exopeptidase digestion on enolase activity. Carboxypeptidase A digestion: enolase, 7.7 mg per ml in 0.06 M Tris chloride buffer containing 5.0 × 10⁻⁵ M Mg⁺⁺, pH 7.75, was digested with carboxypeptidase A at a 1:45 molar ratio of carboxypeptidase to enolase at 30°C. Aminopeptidase M digestion: enolase, 9.4 mg per ml in 0.06 M potassium phosphate buffer containing 6.0 × 10⁻⁵ M Mg⁺⁺, pH 6.9, was digested with 0.1 mg of aminopeptidase M per mg of enolase, at 30°C. In both cases, samples of 15 to 25 µl from both the digestion mixtures (△) and from the digestion-free controls (●) were removed at timed intervals and diluted with the Tris-Mg⁺⁺ assay buffer for activity determinations.
Amino acids released in aminopeptidase digestion of enolase

In Experiment 1, digestion is with 0.18 mg of leucine aminopeptidase per mg of enolase in 0.1 M Tris chloride, 0.01 M Mg\(^{2+}\) buffer, pH 8.4, at 30° for 20 hours. In Experiment 2, digestion is with 0.05 mg of aminopeptidase M per mg of enolase in 0.05 M Tris chloride, 0.01 M Mg\(^{2+}\) buffer, pH 8.4, at 30° for 20 hours. In Experiment 3, digestion is with 0.1 mg of aminopeptidase M per mg of enolase in 0.1 M potassium phosphate, 6 X 10\(^{-3}\) M Mg\(^{2+}\) buffer, pH 6.9, at 30° for 20 hours. All of the values are corrected for aminopeptidase self-digestion which gave background values as high as 0.03 pmole in these experiments. Denaturation of enolase was accomplished by prolonged heating at 100°. In all digestions, parallel experiments included pancreatic ribonuclease A and oxidized insulin as substrates for the aminopeptidases. Ribonuclease was found to be completely resistant to leucine aminopeptidase, and only trace amounts of lysine were released by aminopeptidase M. Oxidized insulin on the other hand was extensively digested by both peptidases.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Native</td>
<td>Denatured</td>
<td>Native</td>
</tr>
<tr>
<td></td>
<td>pmole/µ mole yeast enolase</td>
<td>pmole/µ mole yeast enolase</td>
<td>pmole/µ mole yeast enolase</td>
</tr>
<tr>
<td>Aspartic</td>
<td>0.70</td>
<td>0.22</td>
<td>1.34</td>
</tr>
<tr>
<td>Threonine</td>
<td>Trace</td>
<td>0.32</td>
<td>0.30</td>
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<td>Serine and amides</td>
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<td>0.41</td>
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<td>Glutamic</td>
<td>0.38</td>
<td>0.23</td>
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<tr>
<td>Proline</td>
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<td>0</td>
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<tr>
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<td>1.08</td>
<td>0.47</td>
<td>1.85</td>
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<td>Alanine</td>
<td>0.03</td>
<td>0.75</td>
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<td>Histidine</td>
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<td>N.D. N.D.</td>
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<tr>
<td>Total</td>
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<td>4.2</td>
<td>18.4</td>
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</table>

\(a\) N.D., not determined.

Aminopeptidase Digestion—These experiments were conducted with two control substrates, ribonuclease A as a protein resistant to aminopeptidase action in the absence of endopeptidases and oxidized insulin as a substrate readily attacked by the aminopeptidases. As the results in Table II show, leucine aminopeptidase had no effect on native enolase. In parallel experiments, ribonuclease A was also resistant, whereas oxidized insulin was degraded extensively, giving nearly complete release of some of the neutral amino acids. Denatured enolase was also a poor substrate for leucine aminopeptidase, although some amino acid release was observed in this case. Aminopeptidase M, which has been shown to have highest activity toward amino-terminal alanine (20), did give somewhat better amino acid release from native enolase, but again even prolonged incubation gave only submolar quantities of any one amino acid. Denatured enolase was also here a better substrate, but even under these conditions with the use of high aminopeptidase concentration and deliberately denatured enolase, the total amino acid release is only 10% of that reported in the literature (9). Again, the specificity and activity of the aminopeptidase was checked in parallel experiments against ribonuclease A and oxidized insulin. Only small quantities of lysine were released from the former, whereas the latter was extensively degraded. It may be interesting to note that the results reported in Table II in a qualitative manner not only confirm the alanine amino terminus of enolase, but also are consistent with the proposed amino-terminal sequence of Ala-Gly-Val-Asp-(5).

The conclusion that must be drawn from these experiments is that pure yeast enolase is resistant to endopeptidase-free aminopeptidases and also to endopeptidase-free carboxypeptidases after the initial release of the carboxyl-terminal leucine. Based on the rather indirect support of the above experiments with denatured or impure enolase and with carboxypeptidase B, which was subsequently shown to contain endopeptidase activity, we would like to suggest that the previous results showing extensive amino acid release upon exopeptidase digestion of enolase were due to impurities in either the substrate or the exopeptidases. It is perhaps significant to this suggestion to note the tremendous improvements in analytical techniques in protein chemistry over the last several years. These developments have led to a 20% improvement in the purity of yeast enolase and have also permitted a very precise determination of the purity of exopeptidases (24-26), which was not possible when the initial experiments were carried out.

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Studies on Yeast Enolase: QUANTITATIVE END GROUP ANALYSES AND THE EFFECT OF EXOPEPTIDASE DIGESTION
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