THE DEGRADATION OF YEAST ENOLASE WITH LEUCINE AMINOPEPTIDASE AND CARBOXYPEPTIDASE WITHOUT CHANGE IN ENZYMIC ACTIVITY; N- AND C-TERMINAL RESIDUES OF THE ENZYME

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SUMMARY

1. The earlier finding[5] that enolase consists of a single chain having alanine as the N-terminal amino acid has been confirmed by the phenylisothiocyanate method. The only C-terminal amino acid in the enzyme is leucine, as shown by the carboxypeptidase method. Enolase *a* and *b* do not differ with respect to N- or C-terminal groups.

2. It has been found that the enolase molecule can be extensively degraded with leucine aminopeptidase or carboxypeptidase without loss of activity. Not only the velocity but also the extent of digestion achieved is dependent on the molar ratio between peptidase and enolase. Maximally, about 150 residues can be removed from either end.

3. Some implications of the results for the study of the structure of enzymes are discussed.

INTRODUCTION

It is now a well-established fact that the intact protein molecule is not necessary for the catalytic activity of several enzymes[1]. We have recently reported[2] that about 100 amino acid residues can be removed from the N- or C-terminal end of yeast enolase without change in enzymic activity by digestion with leucine aminopeptidase or carboxypeptidase. The present communication gives a detailed account of an extension of these studies.

As in the case of mercuripapain[3,4], it has been found that the extent of digestion is dependent on the ratio of exopeptidase to substrate (enolase), and with a higher ratio than used previously[2] as many as 150 residues can be removed from either end of the molecule without loss of activity. Several types of evidence are presented showing that the amino acids liberated are derived from the enolase molecule and not from some other constituent in the reaction mixture.

The determination of the N- and C-terminal residues is also described herein. The N-terminal sequence of the enzyme has been reported elsewhere[5]; the present results, obtained by a different method, confirm the previous findings on the nature

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of the N-terminal amino acid (alanine) and the number of peptide chains. Only one C-terminal amino acid (leucine) has been found.

EXPERIMENTAL

Preparation of yeast enolase

The enolase used for end-group determinations was purified by the chromatographic procedure described previously[6]. Since enolase prepared in this way contains about 5 % impurity, the enzyme used for the degradation experiments was purified further by zone electrophoresis[6]; the enzyme so obtained appears homogeneous according to several criteria[6]. Enolase activity was measured as described earlier[7].

Determination of the N-terminal residue

The N-terminal amino acid was determined by a recent modification[8] of EDMAN's phenylisothiocyanate (PTC) method[9]. About 1 ml of an approximately 3 % solution of enolase was mixed with three vol. of pyridine containing 50 μl phenylisothiocyanate/ml; a very fine precipitate was formed. The coupling reaction and subsequent washing was carried out as described by BLOMBÄCK AND YAMASHINA[8] with the modification that 0.2 *N* HCl was added to denature the protein in the first washing with acetone. The cyclization step also followed in detail the procedure of BLOMBÄCK AND YAMASHINA[8]. The phenylthiohydantoin (PTH) amino acids formed were removed by two extractions with 5 ml ethyl acetate, and the extinction at 270 mμ of the combined extracts was measured in the Beckman DU spectrophotometer. The extract was then dried *in vacuo* at room temperature, and the PTH derivatives dissolved in a small volume of acetone–ethyl acetate (1:1) for chromatography. The chromatographic identification was carried out in two systems described by SJÖQUIST[10], the so-called system A and a modification[11] of system C.

Determination of the C-terminal residue

The carboxypeptidase method[12] was used for the identification of the C-terminal amino acid. A 0.7 % solution of enolase in water was shaken with Dowex-50 in the H^+ form (0.5 g/ml solution) to remove free amino acids (*cf.* ref.[5]). After removal of the Dowex by centrifugation, the enolase solution was incubated at room temperature with different molar ratios (1:100 and 1:1000) of carboxypeptidase dissolved in 10 % LiCl[12]. The pH during the digestion was adjusted to about 8.5 by addition of 0.1 *N* NaOH. After 15 min the reaction was stopped by addition of Dowex-50 in the H^+ form (0.5 g/ml solution). The resin was centrifuged down and washed 4 times with 5 volumes of water. The adsorbed amino acids were then eluted with 3 ml of 7 % ammonia, and the solution taken to dryness in a vacuum desiccator. The amino acids were dissolved in a small volume of water and identified by paper chromatography. Two systems were used: (1) *n*-butanol–glacial acetic acid–H_2O (4:1:5), and (2) ethyl methyl ketone–acetone–H_2O (15:5:3).

Digestion of enolase with leucine aminopeptidase and carboxypeptidase

The leucine aminopeptidase (LAP) was prepared from swine kidney as described by HILL AND SMITH[13]. The activity of the enzyme was measured under the same conditions as used by HILL AND SMITH[13,14] (0.05 *M* L-leucinamide in tris (hydroxy-

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methyl) aminomethane–HCl buffer, pH 8.5, containing 0.005 M $MnCl_2$). In all experiments described herein, a preparation having a first-order proteolytic coefficient (C_1) of 90 has been used; however, most preparations yielded a lower C_1 (around 50). The molecular weight of LAP was assumed to be 300,000[15].

The carboxypeptidase (CP) used was a commercial preparation (Worthington Biochemical Corp.) of 3 times crystallized enzyme. A solution of 10% LiCl was used to dissolve the enzyme[12].

The following procedure was used in the degradation experiments: The enzyme was dissolved in 0.005 M $MgSO_4$. Insoluble material was removed by centrifugation, and the concentration (about 10 mg/ml) was determined from the extinction at 280 mμ[16] measured in the Beckman DU spectrophotometer. The pH of the solution was adjusted to 8.0–8.5 by addition of 0.1 N NaOH, and a few drops of toluene were added to minimize the possibility of bacterial contamination. When the solution had reached the temperature of the experiment, a given amount of LAP or CP was added. Aliquots of the incubation mixture were removed at regular intervals for the determination of the amount of amino acids liberated. The protein in the aliquots were precipitated by addition of trichloroacetic acid to a final concentration of 5%, and the amino acids were determined by the ninhydrin method of MOORE AND STEIN[17]. The enolase activity was also measured at regular intervals (5 μl aliquots).

RESULTS

N- and C-terminal residues

Qualitatively, alanine was found to be the only N-terminal amino acid in enolase; not even traces of other end-groups could be detected, as is seen in Fig. 1. The same end group was found to be present in enolase *b* as in enolase *a*. The yield of PTH-alanine was 0.85–0.90 mole/mole of enolase in all experiments. For unknown reasons, it was impossible to detect the N-terminal residue in enolase by FRAENKEL-CONRAT's[18] paper-strip modification of EDMAN's method.

The main difficulty in determining the C-terminal residue was the fact that "pure" enolase contains some rather strongly bound free amino acids. However, these could be successfully removed with Dowex-50 (see EXPERIMENTAL), as is seen from the blank in Fig. 2. This figure also shows the chromatographic identification of the amino acids liberated by two different concentrations of CP (as well as of LAP). It is seen that essentially one amino acid is liberated by CP under the conditions employed, but chromatography in the system used (1) cannot reveal whether this is leucine or isoleucine. However, by chromatography in system 2, it was possible to show that the end-group is leucine. No difference between enolase *a* and *b* could be detected. Since CP attacks different peptide bonds at highly varying rates, a quantitative endgroup determination by this method is complicated, and no attempt at such quantitation was made.

Degradation with exopeptidases

Experiments with varying temperature and concentration of proteolytic enzyme have been performed. The duration of the experiments has never been allowed to exceed 24 h in order to minimize nonspecific losses of activity (bacterial contamination, thermal instability, etc.). Proper blanks (enolase without peptidase, and vice

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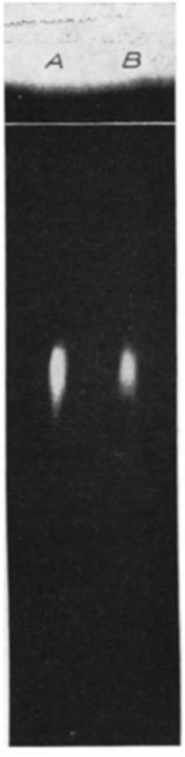

Fig. 1. Chromatography of PTH-alanine (A) and of PTH-derivatives derived from enolase (B). Paper: starch-treated Whatman No. 1; solvent: heptane–pyridine (7:3).

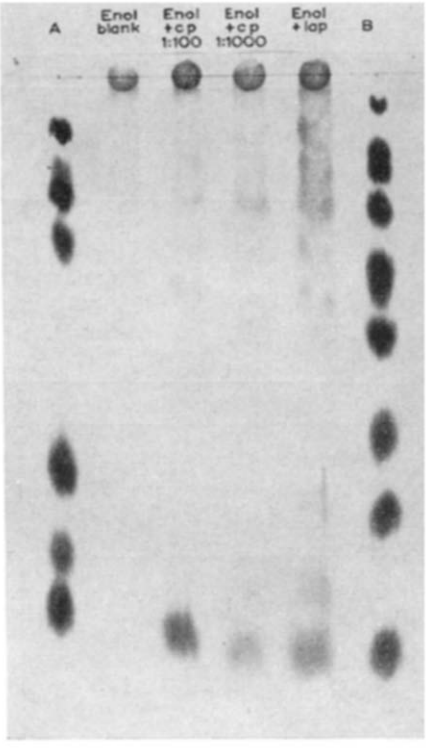

Fig. 2. Chromatography of amino acidsderived from enolase by digestion with CP (and with LAP). The samples are, from left to right: amino-acid mixture A (lysine, aspartic acid, glycine, threonine, valine, tryptophan, phenylalanine, leucine), enolase blank, enolase + CP in molar ratio 100:1, enolase + CP in molar ratio 1000:1, enolas e + LAP, and amino-acid mixture B (cystine, histidine, arginine, serine, glutamic acid, alanine, tyrosine, methionine, isoleucine). Paper: Whatman No. 1; solvent: *n*-butanol–glacial acetic acid–H_2O (4:1:5).

versa) have always been included. Solutions of LAP or CP, as well as of enolase at 10°, have given constant ninhydrin values. At higher temperatures, the enolase solutions have yielded slight increases in ninhydrin color, but all values of amino acids liberated given here have been correspondingly reduced.

The results reported earlier[2] were all performed with a molar ratio between peptidase and enolase of 1:30. Additional experiments with this ratio of enzymes at 22° and 34° have confirmed that between 50 and 100 amino acids are liberated in 24 h by both exopeptidases. However, as in the case of mercuripapain, it was found that considerable increases in the extent of digestion can be achieved by the use of higher ratios, as is seen in Table I. For example with a molar ratio of 1:10, and temperatures of 10°, 22°, or 34°, both LAP and CP can liberate about 150 residues without appreciable loss in enolase activity. Experiments showing this are illustrated in Figs. 3 and 4. The spread in the activity values is considerable, but they must still all be considered to lie within the errors of measurements, which are large (about ± 10%) under the conditions used (high blank extinction, 5 µl enzyme

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TABLE I

THE EFFECT OF THE MOLAR RATIO BETWEEN EXOPEPTIDASE AND ENOLASE ON THE EXTENT OF DIGESTION

Exopeptidase used	*Molar ratio (peptidase: enolase)*	*Temp. (°C)*	*Digestion time (h)*	*Maximum no. of amino acid residue liberated (leucine equivalents/ mole of enolase)*
LAP	1:30	22	22.5	92
LAP	1:12.5	34	10.7	153
LAP	1:10	10	24.0	133
CP	1:25	34	11.0	96
CP	1:10	34	11.0	158, 185
CP	1:5	34	11.0	138

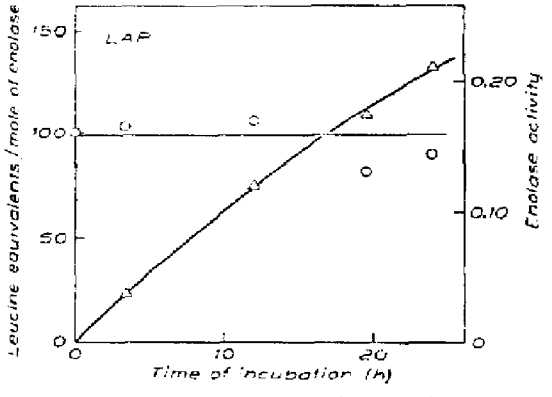

Fig. 3. Activity of enolase (○) and amount (leucine equivalents) of amino acids (△) liberated per mole of enolase during incubation with LAP. Temperature: 10°; molar ratio (LAP/enolase): 1:10.

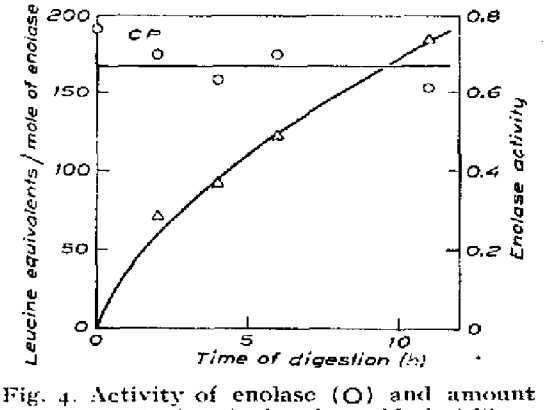

Fig. 4. Activity of enolase (○) and amount (leucine equivalents) of amino acids (△) liberated per mole of enolase during incubation with CP. Temperature: 34°; molar ratio (CP/enolase): 1:10.

samples, long duration of experiment). While the amino acid curves are still quite steep around 150 liberated residues, it has been impossible to go far beyond this point of digestion, even by increasing the molar ratio to 1:2.5 or 1:1.

Several criteria have been used to show that the increase in ninhydrin color observed is due to a degradation of enolase. The blanks used have already been mentioned. By paper chromatography it was shown that the color increase was due to amino acids. In the experiments with LAP, the N-terminal residues after digestion were determined. While the starting material gave only alanine (see above), the LAP-treated enzyme yielded aspartic acid, serine, and alanine, as well as minor amounts of histidine and glycine. In addition, the sedimentation constant was decreased by about 15%, but since no diffusion measurements were carried out, it was impossible to estimate the actual decrease in molecular size.

DISCUSSION

The results here described fully confirm the previous report[2] that the enolase molecule consists of a single peptide chain having alanine as the N-terminal amino acid. The

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fact that qualitatively the only C-terminal residue detected is leucine is also in accord with the presence of a single chain. Since enolase *a* and *b* have the same end-groups, as well as the same molecular weight[6], but differ in charge, it is likely that changes in amide content account for the microheterogeneity, as has been found in the case of ribonuclease[19]. Titration studies on enolase are in progress and may resolve this question.

Limited proteolytic digestion of enzymes without loss of activity has now been accomplished in a number of cases; the literature has been summarized in other places[2,4]. These findings have led to the formulation of the so-called "junk hypothesis"[20] of enzyme structure, *i.e.* the activity of enzymes is assumed to be associated with an active site, and a good portion of the protein molecule is thought to be unessential for the maintenance of this site. While the generality of this hypothesis remains to be established, it offers a promising approach to the determination of the structure of active sites by chemical means even in rather large enzymes. However, such studies are complicated by the fact that, while all of the primary structure of the protein may not be necessary for activity, this is still generally lost by the destruction of the labile tertiary structure. For example, enolase is completely inactive in 6 *M* urea[2]. It is possible that the metal present in many enzymes may stabilize the structure of the active site, so that metalloenzymes[21] may be more promising objects of study in this respect, and, consequently, we have initiated at this Institute structural investigations on two enzymes containing firmly bound metals (carbonic anhydrase and a phenol oxidase[22]).

While the activity of the degraded enolase molecule is unchanged under conditions of maximal activation (high substrate and Mg^{++} concentration), it is likely that the kinetic parameters as well as temperature and pH stability of the enzyme have been altered, and we are at present investigating the properties of the "fragment" in detail. Studies of the quantities of the various amino acids liberated by the exopeptidases are also in progress.

ACKNOWLEDGEMENT

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EFFECTS OF ILLUMINATION AND OXYGEN SUPPLY UPON THE LEVELS OF PYRIDINE NUCLEOTIDES IN *CHLORELLA* CELLS

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SUMMARY

1. Using intact cells of *Chlorella*, investigations were made on the effect of light on the levels of various forms of pyridine nucleotides, compared with that on the photogenic reducing agent (*R*).

2. No form of pyridine nucleotide was found of which the concentration was affected in the same manner as that of *R*, which has been known to be produced only in the light and eliminated completely in the dark.

3. Under both aerobic and anaerobic conditions, light decreased the level of DPN^+ and increased the level of TPN. Quantitatively, there was a correspondence between these effects.

4. When algal cells were transferred in the dark from anaerobic to aerobic conditions, a marked decrease of DPNH occurred, the levels of DPN^+, TPN^+ and TPNH remaining almost unchanged. The decrease of DPNH in the light (in N_2) was assumed to be brought about by the O_2 produced concomitantly with the photogenic formation of *R* and effecting the transfer of DPNH to some substance other than DPN^+.

5. It was concluded that (a) neither TPNH nor DPNH is the primary *R* and that (b) one of the effects caused by light is the conversion of DPN^+ to TPN.

INTRODUCTION

Since the work of CALVIN and his associates[1,2] it is now generally agreed that the reductive step involved in the photosynthetic cycle is the transformation of phosphoglyceric acid to 3-phosphoglyceraldehyde, an enzymic reaction that is known to

Abbreviations: DPN^+ and DPNH, oxidized and reduced diphosphopyridine nucleotide; TPN^+ and TPNH, oxidized and reduced triphosphopyridine nucleotide; total DPN = DPN^+ + DPNH; total TPN = TPN^+ + TPNH; PN^+ = DPN^+ + TPN^+; PNH = DPNH + TPNH; total PN = total DPN + total TPN.

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