One approach to the general problem of the structure and function of enzymes involves proteolytic hydrolysis with examination of the structure, composition, and catalytic activity of the hydrolysis products. Fragments retaining partial enzyme activity have been obtained from trypsin and pepsin by autolysis (2, 3) and from trypsinogen by peptic digestion (4). Over half of the amino acid residues of pepsin may be removed by aminopeptidase without activity loss (5). Ribonuclease loses activity when a peptide of 20 residues is removed from the NH₂-terminal end, but regains activity when recombined with the peptide (6). Removal of COOH-terminal residues by carboxypeptidase has little effect on the activity of chymotrypsin (7), lysozyme (8), or ribonuclease (9, 10). In contrast, studies in this laboratory, preliminary to the investigations reported herein, (11), showed that treatment of muscle aldolase by crude trypsin or carboxypeptidase readily caused marked activity loss.

The purpose of this paper is to present observations on the striking decrease in aldolase catalytic activity and concomitant change in specificity accompanying release of terminal tyrosine residues by carboxypeptidase digestion.

EXPERIMENTAL

All solutions of enzymes and substrates were prepared in distilled water passed through a mixed ion exchange resin bed (Rohm and Haas Monobed 3) to remove traces of ionic impurities. Water treated in this manner is referred to as ion-low water.

Enzymes and Substrates—Aldolase was a crystalline preparation from rabbit muscle made according to the method of Taylor et al. (12) with minor modifications as reported previously (13).SUCCESSIVE PREPARATIONS WERE MADE AS REQUIRED AND ALL PREPARATIONS WERE RECRYSTALLIZED TWO TO FIVE TIMES AND STORED IN THE PRESENCE OF THE CRYSTALLIZING MEDIUM AT 2-5° UNTIL USED. THE MOLAR CONCENTRATION OF ALDOLASE WAS DETERMINED FROM ITS ABSORBANCE AT 280 mμ (A280/0.1 = mg of aldolase per ml) AND ITS MOLECULAR WEIGHT OF 149,000 (15).

Carboxypeptidase was a twice recrystallized preparation obtained in water suspension from the Worthington Biochemical Corporation. Just before use, carboxypeptidase was brought into solution at 4° in 10% LiCl (16) and adjusted to pH 7.8. Molar concentration of carboxypeptidase was determined from its absorbance at 280 mμ (A280/2.3 = mg of carboxypeptidase per ml) and its molecular weight of 34,400 (16). When amino acid analyses were to be made with carboxypeptidase-aldolase hydrolysates, carboxypeptidase crystals were first washed five to six times in water before solution in 10% LiCl. Carboxypeptidase treated with diethyl-p-nitrophenyl phosphonate according to Harris (8) was kindly supplied by Dr. S. Wada.

Fructose 1,6-diphosphate (fructose-di-P) and fructose 1-phosphate (fructose-1-P) solutions were prepared by removal of divalent cations on suitable ion exchange resins followed by neutralization to pH 7.4; stock solutions were kept frozen when not in use.

Aldolase Activity Assay—Aldolase activity was determined with fructose-di-P as substrate according to the colorimetric method of Sibley and Lehninger (17) as modified by Swenson and Boyer (13). With fructose-1-P as substrate, the same procedure was followed with fructose-1-P at a final concentration of 7 to 8 × 10⁻³ M, and an approximate 150-fold increase of aldolase (about 150 μg per ml final concentration). Specific activities (13) were 45 to 51 for various aldolase preparations with fructose-di-P as substrate and 0.3 to 0.32 with fructose-1-P as substrate.

In some of the work, the assay based on absorption increase at 280 mμ (18) was used, with aldolase at a final approximate concentration of 3 μg per ml. This procedure shows an undesirable lack of linearity of ΔA280 with time. Satisfactory comparative measurements were possible by use of the average change in A280 over the third to sixth minutes. Measurements were made in a Beckman spectrophotometer with the cell compartment temperature controlled by circulating H₂O.

Aldolase activity remaining during the course of a carboxypeptidase digestion was determined with an aliquot diluted with 0.001 M EDTA at pH 7.8 to an appropriate aldolase concentration. Such diluting effectively stopped the carboxypeptidase action as demonstrated by suitable controls in which the carboxypeptidase and aldolase were diluted before mixing.

Colorimetric or ultraviolet absorption assay of degraded aldolase with fructose di-P required an approximate 10-fold increase in enzyme concentration over that used in the respective aldolase assay, whereas with fructose-1-P no increase in enzyme concentration was necessary.

* Supported in part by Research Grant 4930 of the United States Public Health Service, and Grant 1753 of the National Science Foundation. Taken in part from the Ph.D. Thesis of E. R. Drechsler. Much of the experimental work reported herein was carried out in the Department of Agricultural Biochemistry, University of Minnesota, St. Paul. A preliminary report of this work has appeared (1).

† Present address, Department of Science and Mathematics, State University Teachers College, Plattsburgh, New York.
Amino Acid Analysis—Quantitative analyses were performed on supernatant solutions obtained from carboxypeptidase-aldolase incubation mixtures by precipitation of protein with cold trichloroacetic acid at a final concentration of 5%. Protein precipitates were washed with cold 5% CCl₄COOH and the washings and supernatants pooled for analysis.

Amino acid release was determined on supernatants from precipitation with 5% CCl₄COOH. Total amino acid was determined as "leucine equivalents" with the ninhydrin method of Troll and Cannan (19), without prior neutralization of the CCl₄COOH (20). Tyrosine was determined by a modification of the Folin-Ciocalteau method (21). As an independent check, the ultraviolet absorption method of Goodwin and Morton (22) was also used for tyrosine, based on measurement of the absorption at 293 mμ in 0.1 N NaOH, and a molar extinction coefficient of 2930 for tyrosine. For these measurements, the CCl₄COOH was first neutralized and the NaOH adjusted to a final calculated concentration of 0.1 N.

Qualitative amino acid analyses of carboxypeptidase-aldolase incubation mixtures were made by the one-dimensional, descending paper chromatographic technique on Whatman No. 1 or No. 4 paper. Solvent systems used were: 4:1:5 n-butanol-acetic acid-water; 12:1:1 n-butanol-formic acid-water; water saturated phenol containing 0.005% 8-hydroxyquinoline (23) and 5:3:1:1 n-butanol-2-butanone-water 15% ammonia (24). Spots were visualized with a ninhydrin spray, 0.25% in acetone. Standard amino acids were included as markers. When necessary CCl₄COOH was removed from aliquots of supernatants by other extraction, followed by adsorption of the free amino acids and elution on to from Amberlite IR 120 cation exchanger resin (Rohm and Haas), 20 to 40 mesh, in the hydrogen form. Aliquots were also prepared for paper chromatography by direct determination of the carboxypeptidase action with IR 120 resin and elution of the free amino acids therefrom, according to published procedures (8).

To insure the absence of interfering material or extraneous amino acids from the starting solutions of aldolase, the solutions were exhaustively dialyzed in the cold against 10⁻² M EDTA at pH 7.8 and then the dialyzed protein solution was passed through a mixed ion exchange resin bed (Rohm and Haas Monobed 1). Nearly quantitative recovery of activity and protein was obtained after such treatment.

Optical rotation measurements were made, through the cooperation of Dr. John Schallman of the Department of Chemistry, with a Rudolph precision photoelectric polarimeter, with the use of a 1-cm micro polarimeter tube.

RESULTS

Loss of Aldolase Activity in Presence of Carboxypeptidase—When 1 to 1.5% solutions of aldolase were incubated with carboxypeptidase in 10⁻² M EDTA, 0.5 to 1.5% LiCl, pH 7.8 to 8.0, a rapid but incomplete loss of aldolase activity resulted. Controls without carboxypeptidase showed little or no loss when incubated under similar conditions. The rate, but not the extent of activity loss, was changed by variation of the aldolase:carboxypeptidase ratio. At aldolase:carboxypeptidase ratios of 1000, aldolase underwent activity loss within a 5-minute period to a product retaining approximately 7% of the original activity. That the presence of this residual aldolase activity did not result from decreased potential carboxypeptidase activity, and that the activity loss was not due to trypsin or chymotrypsin contamination in the carboxypeptidase, was shown by the following experiments:

(a) Use of more carboxypeptidase so as to decrease the mole ratio of aldolase:carboxypeptidase to as low as 20, or increasing the time of incubation up to 3 hours, did not result in further activity loss.

(b) Fresh aldolase underwent rapid degradation when added to an incubation mixture in which the original aldolase had lost some 93% of its activity.

(c) Carboxypeptidase and carboxypeptidase which had been treated with diethyl-p-nitrophenylphosphonate to inactivate endopeptidases gave entirely analogous activity losses.

The activity loss upon carboxypeptidase treatment was readily reproducible. In the course of these experiments, similar residual activities upon carboxypeptidase digestion have been obtained with at least seven different aldolase preparations varying from crude to highly purified.

Qualitative Identification of Amino Acids Released—Paper chromatography of aliquots of carboxypeptidase-aldolase incubation mixtures, prepared for chromatography by either of the procedures mentioned in "Experimental," showed that the first and only amino acid appearing in significant amounts during the degradation of aldolase to minimum activity was tyrosine. For example, an experiment was performed in which samples of carboxypeptidase-aldolase incubation mixtures were taken when residual activity was at 16, 8, and 7% of initial aldolase activity. Samples were prepared for chromatography by direct termination of the reaction with IR 120-H resin. Only tyrosine appeared on the chromatogram of the 16% activity sample. In addition to the tyrosine spot, a very faint spot migrating with an Rₕ near that of alanine began to appear in the 8% sample and became more intense in the 7% sample. With continued incubation or with higher concentrations of carboxypeptidase, two moles of alanine per mole of aldolase appeared accompanied by traces of other amino acids tentatively identified as histidine, serine and phenylalanine using the solvent systems given in the experimental section.

The identity of the first amino acid released as tyrosine was established by comparison of the Rₕ values with authentic tyrosine in the solvent systems mentioned above, by the characteristic slate gray color obtained with a ninhydrin-collidine spray agent, the streaking and tailing in the n-butanol-acetic acid-water solvent system as reported by Gladner and Neurath (7), by the qualitative identity of the ultraviolet absorption spectrum with authentic tyrosine, by the production of color with the Folin reaction, and by the identification as tyrosine by the specific test based on reaction with α-nitroso-β-naphthol (25). The absence of tryptophan was evident from the chromatographic studies and was further demonstrated by the specific test of Spies and Chambers (26). Good agreement was obtained in quantitative measurement of tyrosine released based on either the ultraviolet absorption or reaction with the Folin reagent.

"Zero time" controls or tests with aldolase alone revealed slight amounts of ninhydrin-positive material with an Rₕ in the vicinity of glycine if aldolase was not pretreated with MB-1 resin before incubation with carboxypeptidase. Extensive washing of carboxypeptidase in distilled water before its solution in 10% LiCl eliminated faint spots in the vicinity of cysteine. With these precautions, appearance of nonprotein ninhydrin reactive material was entirely dependent upon carboxypeptidase digestion.
Correlation of Amino Acid Release with Activity Loss—For quantitative determination of tyrosine release as correlated with activity loss, appropriate samples of dialyzed and resin-treated aldolase were incubated with carboxypeptidase and aliquots withdrawn at various time intervals for activity assay and free tyrosine determinations by use of the Folin reagent. Results of such experiments are shown in Fig. 1. It is evident that a nearly linear relationship exists between loss of aldolase activity and release of tyrosine, and that maximum activity loss is reached when tyrosine release is at a maximum of 3 moles tyrosine per mole of aldolase.

The relation between total amino acid released and activity loss was evaluated by use of quantitative ninhydrin assay. A representative experiment is as follows. Aldolase was incubated at an aldolase:carboxypeptidase ratio of 20 and attained minimum activity within 1 minute with release of 3 moles of tyrosine per mole of aldolase. However, the total amino acid released in this sample as measured by the ninhydrin reaction was 3.4 leucine equivalents per mole of aldolase. After incubation for 5 minutes, analysis showed that tyrosine release remained constant but that total amino acid had reached a value of 4.5 leucine equivalents per mole of aldolase. Under the conditions used in the ninhydrin assay, authentic tyrosine consistently gave a value of 0.84 leucine equivalent. Of the total 4.5 leucine equivalents found per mole of aldolase, tyrosine would thus account for 2.5, leaving 2.0 “extra” leucine equivalents per mole of aldolase not accounted as tyrosine. Clearly 5 amino acid residues are readily liberated from aldolase by carboxypeptidase, and only three of these are tyrosine.

Measurements were made of the total amino acid and of tyrosine release during the early stage of the degradation when minimum activity had not yet been reached to give additional information as to the order of release of the amino acids. The relationship of total amino acid to tyrosine release under variable incubation conditions is shown in Fig. 2. It is seen that tyrosine is the only amino acid released during the degradation of aldolase to the minimum 7% activity. The results in Fig. 2, in harmony with the qualitative paper chromatographic experiments given earlier, show that only after minimum activity has been reached does other amino acid begin to appear. Quantitatively, the release of this amino acid approaches a limit of two leucine equivalents per mole of aldolase under the most drastic conditions used, i.e. incubation at an aldolase:carboxypeptidase ratio of 20.

Crystalline Active Degraded Aldolase—The establishment of a constant residual activity remaining after carboxypeptidase action on aldolase indicated the probable presence of a new molecular species. This activity still showed characteristics attributable to protein. In experiments with solutions of maximally degraded aldolase, the residual activity was completely lost when subjected to boiling water bath temperatures for 5 minutes. Activity was fully retained upon standing at room temperatures for periods up to several hours, at 30° for 2 hours, or at 2-4° for several days. Incubation for 5 minutes at 30° with either 5 × 10^{-4} M Cu\textsuperscript{2+} or Ag\textsuperscript{+} led to complete loss of activity. When the solution was dialyzed in the cold against 10^{-3} M EDTA, pH 7.4, for periods up to 48 hours, no activity could be detected in the dialysate whereas practically all activity was recovered within the dialyzed solution.

Initial trials with ammonium sulfate fractionation showed that all activity and protein could be precipitated at 65% ammonium sulfate.
A summary of the crystallization and recrystallization is given in Table I.

**Physical and Chemical Properties of Aldolase and Degraded Aldolase**—Ultracentrifuge runs were made with approximately 1% solutions of crystalline aldolase or degraded aldolase under conditions closely similar to those used by Gralén (27). Both aldolase and degraded aldolase showed homogeneous schlieren patterns with sharp peaks throughout the duration of a 90 minute run at 59,780 r.p.m. When a combined sample of aldolase and degraded aldolase was run in the ultracentrifuge, only one sharp peak was observed throughout the duration of a 112 minute run. Immediately before this run it was shown that the aldolase sample incubated for 1 hour at 30° with a large excess of the degraded aldolase underwent no loss of activity. Thus the degraded aldolase preparation was free from carboxypeptidase, and native aldolase would be expected to remain unchanged in the mixture.

The optical rotation of aldolase was measured before, during, and after its conversion to degraded aldolase by carboxypeptidase to determine if any major structural changes had occurred in the aldolase molecule during conversion to degraded aldolase. The results are summarized in Table II, where it is seen that little or no change occurs in the specific rotation of aldolase during its conversion to degraded aldolase.

The chemical environment or hydrogen bonding of tyrosine residues in a protein may be partially revealed by examination of absorption spectra at different pH values (28, 29). Native aldolase shows a marked difference in absorbancy at pH 6.8 and 1.3, as shown by the difference absorption spectrum given in Fig. 3. The difference spectrum results from a lowering of the absorbancy at acid pH accompanied by a shift of the maximum from 280 to 277 mμ. Possible contributions to the spectral change may be that at pH 7.3 the tyrosine residues are extensively involved in hydrogen bonding or in ion-dipole interactions with closely adjacent groups (28, 29), or that the tyrosine residues may lie in hydrophobic regions created by aliphatic side

### Table I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total* protein</th>
<th>Initial specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial aldolase</td>
<td>244</td>
<td>100</td>
</tr>
<tr>
<td>Incubation solution of degraded aldolase</td>
<td>242</td>
<td>7.0</td>
</tr>
<tr>
<td>First crystalline degraded aldolase</td>
<td>312</td>
<td>7.0</td>
</tr>
<tr>
<td>Once recrystallized degraded aldolase</td>
<td>178</td>
<td>6.7</td>
</tr>
<tr>
<td>Twice recrystallized degraded aldolase</td>
<td>172</td>
<td>6.9</td>
</tr>
<tr>
<td>Three times recrystallized degraded aldolase</td>
<td>158</td>
<td>7.0</td>
</tr>
</tbody>
</table>

* Both aldolase and degraded aldolase concentrations were determined from absorption at 280 mμ with the value $A_{280/0.91}$ = mg of protein per ml (13) for aldolase, and $A_{180/0.94}$ = mg of protein per ml for degraded aldolase. The latter value is estimated from the expected contribution of tyrosine and tryptophan residues to the $A_{280}$ value based on the amino acid composition and loss of 3 tyrosine residues per mole.

### Table II

**Specific optical rotation of aldolase and degraded aldolase**

The rotation at 24°, 5471 A, of a sample of dialyzed, resin-treated aldolase, 26.3 mg per ml in 10⁻² M EDTA solution, adjusted to pH 7.8 and 1% in LiCl was determined. Then carboxypeptidase in 10⁻² M LiCl, pH 7.8, was added to give an aldolase:carboxypeptidase mole ratio of 810. Dilution of the aldolase solution by carboxypeptidase or contribution of carboxypeptidase to rotation was negligible. Incubation proceeded at 24° over a period of 50 minutes while the rotation was followed. After 90 minutes, a sample was withdrawn for activity assay.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Initial aldolase activity</th>
<th>Specific rotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldolase</td>
<td>100</td>
<td>$-26.1^o \pm 0.2^o$</td>
</tr>
<tr>
<td>50 min with carboxypeptidase</td>
<td>7</td>
<td>$-26.6^o \pm 0.2^o$</td>
</tr>
</tbody>
</table>

The optical rotation of aldolase was measured before, during, and after its conversion to degraded aldolase by carboxypeptidase to determine if any major structural changes had occurred in the aldolase molecule during conversion to degraded aldolase. The results are summarized in Table II, where it is seen that little or no change occurs in the specific rotation of aldolase during its conversion to degraded aldolase.

The chemical environment or hydrogen bonding of tyrosine residues in a protein may be partially revealed by examination of absorption spectra at different pH values (28, 29). Native aldolase shows a marked difference in absorbancy at pH 6.8 and 1.3, as shown by the difference absorption spectrum given in Fig. 3. The difference spectrum results from a lowering of the absorbancy at acid pH accompanied by a shift of the maximum from 280 to 277 mμ. Possible contributions to the spectral change may be that at pH 7.3 the tyrosine residues are extensively involved in hydrogen bonding or in ion-dipole interactions with closely adjacent groups (28, 29), or that the tyrosine residues may lie in hydrophobic regions created by aliphatic side.
It should be noted, however, that the maximum absorbancy difference (Fig. 3) amounts to a εₓ of 800 per tyrosine residue. This is much larger than noted for other proteins or simple tyrosine derivatives (28, 29), and suggests that change in absorbancy of tryptophan residues may contribute considerable to the difference spectrum.

The difference absorption technique was also used to compare aldolase and a carboxypeptidase digest of aldolase in a manner similar to that used for insulin and its tryptic digest (29). A solution of aldolase, containing 1.6 mg per ml in 10⁻² M EDTA, 1% LiCl, at pH 7.8, was compared to a similar, blank solution to which carboxypeptidase had been added at an initial aldolase: carboxypeptidase ratio of 210. The relative absorbancy at 287 μ of the enzyme-treated aldolase, as measured against the unaltered aldolase, decreased slightly over a few minute period, and then remained constant. The total increment, ΔAₓ₁₈₅, amounted to 0.018. Activity assay confirmed that residual activity had been reached. The results are indicative of a change in hydrogen bonding or chemical environment of one or more of the tyrosine molecules released.

Changes in the properties of the aldolase molecule upon carboxypeptidase degradation might also be revealed by measurement of sensitivity to heat denaturation. The data of Fig. 4 show the effect of time of heating at 60° under identical conditions on the catalytic activity as measured in the usual assay with fructose-di-P at 30°. The degraded aldolase is definitely more resistant to loss of activity upon heating than aldolase.

Another approach which may reveal changes in properties and structure is measurement of the rate and extent of reaction of various chemical groups on the protein. The rate and extent of reaction of aldolase and degraded aldolase with p-mercuribenzoate were compared under the same conditions as used by Swenson and Boyer (19) for aldolase. Within experimental error, the rate and extent of reaction of aldolase was the same as that for degraded aldolase.

Catalytic Activity with Fructose-1-P and Fructose-di-P—The ability of rabbit muscle aldolase to cleave fructose-1-P as well as fructose-di-P has been reported (31-33). Results of experiments to assess the effect of carboxypeptidase degradation on the fructose-1-P activity of aldolase are shown in Table III. These results show that fructose-1-P activity is not lost by carboxypeptidase degradation and that highest recoveries of fructose-1-P activity seem to be reached at points somewhat before minimum fructose-di-P activity is reached. These results may reflect the existence of a transient molecular species possessing enhanced fructose-1-P activity. In similar experiments the ratios of activity with the two substrates have been somewhat variable, but with the consistent finding that fructose-1-P activity was always recovered in excess of 100% of the initial fructose-1-P activity, providing fructose-di-P activity had approached maximum degradation. No change in fructose-1-P activity was noted unless the loss of activity toward fructose-di-P had been extensive.

Energy of Activation for Catalysis by Aldolase and Degraded Aldolase—The effect of temperature on the rate of cleavage of fructose-1-P by aldolase or degraded aldolase was studied over the range of 16 to 37°, with the ultraviolet assay method. Assays were made with 0.004 M fructose-di-P, far in excess of the Km; this obviates change in Km with temperature as a factor modifying measured activation energy. Possible change in pH activity relationships with temperature were not controlled. Plots of 1/ν against 1/T according to the integrated form of the Arrhenius equation for both aldolase and degraded aldolase gave curves very slightly concave downwards, indicating a gradual change in activation energy with temperature. From the slopes of the best straight lines through the points, an activation energy of close to 16,000 calories per mole was calculated for both aldolase and degraded aldolase.

Michaelis Constants and Maximum Velocities for Fructose-di-P and Fructose-1-P—The effect of substrate concentration on
The rate of cleavage of fructose-di-P and fructose-1-P by crystalline aldolase and degraded aldolase was studied. The data are plotted according to the method of Eadie (34) in Fig. 5. Calculated values for the Michaelis constants and maximum velocities are given in Table IV. The value for the \( K_m \) of fructose-di-P with degraded aldolase is less accurate than the other values because sensitivity of the assay procedure made initial velocity measurements difficult at the requisite lower substrate concentrations. The accuracy does suffice to establish clearly that the \( K_m \) of fructose-di-P for degraded aldolase is less than that for native aldolase. Similarly, the \( K_m \) for fructose-1-P is less for degraded than native aldolase, although much higher for both enzymes than the \( K_m \) for fructose-di-P.

**TABLE IV**

<table>
<thead>
<tr>
<th>Kinetic parameters for aldolase and degraded aldolase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose-di-P</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td><strong>Aldolase</strong></td>
</tr>
<tr>
<td><strong>Degraded aldolase</strong></td>
</tr>
</tbody>
</table>

\* Maximum velocities at a given enzyme concentration are relative to that for fructose-di-P at 100; the observed \( \Delta A_{240} \) per minute with fructose-di-P was 0.031.

**TABLE V**

<table>
<thead>
<tr>
<th>Aldolase and degraded aldolase activities in combined presence of fructose-di-P and fructose-1-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
</tr>
<tr>
<td>Fructose-di-P</td>
</tr>
<tr>
<td>Fructose-1-P</td>
</tr>
</tbody>
</table>

\| Expected if not competitive: 22.

Discussion

Aldolase is unique among enzymes thus far studied in that enzyme activity is largely lost with the loss of a few amino acids at or near COOH-terminal ends of peptide chains. Of particular interest is the retention of partial activity and change in specificity accompanying carboxypeptidase digestion. The demonstration that tyrosine is the first amino acid to be released shows it to be a C-terminal amino acid.

It has been reported (36) that aldolase has two NH\(_2\)-terminal amino acids. On such basis, the simplest conclusion to be drawn from the present study is that tyrosine occupies the COOH-terminus of each of the two peptide chains of aldolase. The third tyrosine residue released from aldolase could have been COOH-terminal on a third peptide chain or could have been penultimate in one of the two chains. However, as is well recognized (37), use of carboxypeptidase for COOH-terminal analysis of proteins can give evidence for assigning an amino acid to the COOH-terminal position only in case one peptide chain is involved. Unequivocal assignment of COOH-terminal position requires corroboratory evidence by chemical methods.\* The correlation of all activity loss with the release of three tyrosine residues, and the appearance of two alanine residues after tyrosine release was complete, suggest that the alanine residues are not involved in aldolase catalytic activity.

The ability of carboxypeptidase to liberate amino acids readily from aldolase is clearly limited to the release of three tyrosine and two additional amino acids from the aldolase molecule. The resistance to further digestion could reflect nonconformity to the specificity requirements of carboxypeptidase. Proline residues in the terminal or penultimate position as well as presence of lysine or arginine residues limit carboxypeptidase action (20, 37, 38). Recent studies by Dr. A. Kowalsky (38) in this laboratory, with the use of a combined O\(_{18}\)-carboxypeptidase procedure, have shown that all three tyrosine residues are C-terminal.
The high recovery of crystalline degraded aldolase shows that aldolase was quantitatively converted to degraded aldolase. The constant specific activity during isolation and repeated recrystallization gives strong evidence that observed residual activity is a property of the entire recovered crystalline product, and not the result of the fortuitous presence of a constant contamination originally present in aldolase which had been carried along unaffected by crystallization and recrystallization of aldolase or degraded aldolase or by carboxypeptidase action. Additional evidence for this comes from experiments which showed that a similar level of residual activity was obtained with crude as compared to highly purified preparations of native aldolase.

Several facets of the data show that the transformation of native to degraded aldolase is not accompanied by major structural change. Proteins with very low levorotations are highly folded and formation of a random coil from an a-helix configuration is accompanied by a large increase in levorotation (40, 41). On this basis, the fact that the specific optical rotation of -26.2° for aldolase underwent little or no change during the conversion to degradedaldolase shows that aldolase exists in a highly folded state and undergoes little or no change in structure during the conversion. Lack of structural change is also indicated by the identical rate of reaction of -SH groups of aldolase and degraded aldolase with p-mercuribenzoate. Only roughly ¾ of the -SH groups of aldolase react rapidly with p-mercuribenzoate at neutral pH and room temperature, and relatively low urea concentrations greatly increase reactivity of the -SH groups (18). Thus the reaction rate with p-mercuribenzoate may give a sensitive criterion of structural change.

The analytical value of 41 tyrosine residues per mole of aldolase (42) makes likely the existence of considerable interaction of tyrosyl residues with other groups in the enzyme. Evidence for such interaction is given by the finding of a considerable difference in absorption at 287 μm in acid as compared to neutral solution. The presence of 15 tryptophan residues per mole of aldolase (42) contributes more than the tyrosine to the absorption at 280 μm because of the considerably larger εm of tryptophan. The relatively large spectral change in the 270 to 280 μm region upon acidification of aldolase suggests that the absorption of the tryptophan residues is influenced by accompanying structural changes.

The finding of only a small change in absorption at 287 μm when aldolase was degraded with carboxypeptidase indicated that only a small change had occurred in the environment of aromatic amino acid residues during the conversion to degraded aldolase. This may reflect changes in absorption of the liberated tyrosines. These results support the conclusion that no large structural changes occur in the conversion. Further evidence comes from the identical ultracentrifugal sedimentation behavior of aldolase and degraded aldolase. The removal of amino acid residues during the conversion increased the resistance of the bulk of the folded polypeptide to heat denaturation. This could be a reflection of structural change, or of some promoting effect of the amino acids residues susceptible to carboxypeptidase removal.

The tyrosines removed by carboxypeptidase digestion have a much more critical role in the cleavage of fructose-di-P than of fructose-1-P. Indeed, the similar activities of aldolase and degraded aldolase toward fructose-1-P suggest that catalysis toward this substrate is largely independent of the tyrosines. The present findings do not allow choice between the alternatives that the tyrosine residues promote fructose-di-P cleavage by direct interaction with the substrate during the catalysis or by favoring a particular catalytically more active structure of the protein independent of any direct interaction with the tyrosines and fructose-di-P. The results do rule out any major structural difference between aldolase and degraded aldolase, but obviously only a very minor difference could conceivably markedly alter catalytic properties. One possibility that merits consideration is a direct binding of the tyrosyl residues to the phosphate in position 6 of fructose-di-P. Phosphate, oxygen, and hydroxyl groups may participate in strong hydrogen bonding (43), and interaction of phosphate need not be limited to ionic attractions. Varied and more detailed speculations could be made but do not appear warranted at this time.

The linear relation between activity loss and tyrosine release does not establish whether activity loss may be critically affected by removal of a certain tyrosine residue or result from the random release of the three tyrosine residues per molecule. The linear relationships might result with either "all-or-none" type or a random release of the tyrosines.

That Michaelis constants may not give a measure of enzyme-substrate dissociation constants has become increasingly clear from experimental findings as well as theoretical interpretations. Further, substrates with similar maximum velocities but different Michaelis constants for the same enzyme may not necessarily have different dissociation constants. Thus limited significance can at present be attached to the lower Michaelis constants of degraded as compared to native aldolase for fructose-1-P and fructose-di-P.

The much greater maximum velocity attainable with fructose-di-P obviously shows that the free energy of formation of the activated transition state is less for this substrate with native aldolase than for degraded aldolase. The similar energy of activation means that the ΔH for formation of the activated complex does not change, and thus the entropy of activation for degraded aldolase is less than that for native aldolase.

The finding that fructose-1-P is cleaved by rabbit muscle aldolase is in agreement with previous reports (31-33). The competition of fructose-di-P and fructose-1-P for the same binding site of aldolase or degraded aldolase (Table V) is reasonably expected on the basis of the structural similarities of the two substrates. It has been shown (44) that aldolase is specific for dihydroxyacetone-phosphate, the moesty common to both fructose-di-P and fructose-1-P. The results show that fructose-di-P and fructose-1-P activities of either aldolase or degraded aldolase are the property of only one enzyme. Leuchtardt and Wolf (45) had claimed the existence of both a "fructose-diphosphate aldolase" and a "fructose-1-phosphate-aldolase" in muscle, but in a later report (35) appear to have withdrawn this claim.

SUMMARY

Muscle aldolase is rapidly degraded by carboxypeptidase to a product retaining approximately 7% of the original catalytic activity with fructose 1,6-diphosphate as a substrate. In contrast, activity with fructose 1-phosphate as a substrate is slightly increased. Competition experiments indicate that the same active sites are responsible for catalysis with both substrates.

Amino acid release during carboxypeptidase digestion is limited to three moles of tyrosine per aldolase molecule, followed by
release of two moles of amino acid having chromatographic properties of alanine. Activity loss is proportional to the tyrosine released, and the additional amino acid release does not further reduce the residual activity. Tyrosine groups at or near carboxy-terminal ends of peptide chains thus have a vital role in aldolase activity.

The residual degraded aldolase may be readily recrystallized without change in specific activity. The degraded aldolase is identical with the native aldolase as measured by ultracentrifugation, optical rotation, or rate and extent of reaction of -SH groups with p-mercuribenzoate. Native aldolase in acid as compared to neutral solution shows a marked difference in absorption in the 270 to 280 nm region, probably reflecting changes in the environment of tyrosine and tryptophan residues. Tyrosine release by carboxypeptidase action is accompanied by only a slight change in absorption at 287 nm. These various findings show that little or no change in gross molecular architecture accompanies the degradation. The degraded aldolase is slightly more resistant to heat denaturation than native aldolase.

The Michaelis constants with both fructose 1,6-diphosphate and fructose 1-phosphate are lower for degraded than for native aldolase. Both forms of aldolase show similar heats of activation with fructose 1,6-diphosphate as a substrate. Some implications of the findings with respect to the catalytic mechanism are discussed.

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