Studies on the Carboxyl- and Amino-terminal Residues of Rabbit Muscle Aldolase*

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Many biologically active proteins have been found to contain more than one polypeptide chain, and in most of these proteins the chains appear to be held together in the active structure without the participation of covalent bonds. In these cases relatively mild conditions, presumably too mild to break covalent bonds, will cause dissociation of the protein into its component peptide chains (structural subunits), and the fact that the peptide chains generally can be reassociated to give back the active protein molecule, must mean that the subunit interaction is highly specific. The phenomenon of multichain structure in proteins has many interesting ramifications in protein chemistry as well as in the areas of molecular genetics, protein synthesis, and biological control.

Rabbit muscle aldolase is one of the best documented cases of a multichain protein. The enzyme with a molecular weight of 150,000 can best be described in terms of three individual polypeptide chains, each containing a carboxyl-terminal tyrosine residue (1, 2) and an amino-terminal proline residue (3). The three chains can be dissociated under a number of conditions, giving an apparently homogeneous mixture of chains with a molecular weight of 50,000 (4, 5). Upon removal of the dissociating agent, the chains reassociate and the active enzyme is reconstituted in good yield (4, 5). Digestion of rabbit muscle aldolase with trypsin and chymotrypsin gives a number of peptides which is very close to that which should be expected if the three chains were identical (6). On the other hand, Kowalsky and Boyer (2) have reported that carboxypeptidase A treatment of aldolase gives 3 moles of carboxyl-terminal tyrosine, followed by only 2 moles of alanine, and it thus appears that the three peptide chains may be different with respect to their penultimate residues. To complicate the picture further, Hass and Lewis (7) have recently demonstrated the formation of subunits with a molecular weight of 22,000 from alkali-treated aldolase and have consequently suggested that aldolase contains six polypeptide chains.

It was considered possible that the discrepancy between the results of the end group analysis of aldolase (giving good evidence for three chains) and the physical evidence for six chains might reflect the limitations inherent in the methods of end group analysis, and that other “masked” end groups could be present in the enzyme without being detected by the methods employed. The present work was undertaken with the goal of uncovering any “masked” end groups in aldolase and thereby either confirm the evidence for the three-chain model or obtain support for the six-chain structure. In addition, it was also felt of interest to identify the carboxypeptidase A resistant amino acid residue in one of the chains of aldolase and define the implied difference between the chains in terms of specific amino acid sequences.

EXPERIMENTAL PROCEDURE

Aldolase—Crystalline rabbit muscle aldolase was obtained from the Sigma Chemical Company (crystalline suspension, lots A92B-084 and A82B-089). The enzyme was shown to be homogeneous in the ultracentrifuge. All protein concentrations were determined spectrophotometrically by using a value of $E^{1}^{{1 cm}} = 0.91$ at 280 mµ (8). The coupled enzyme assay described by Richards and Rutter (9) for measuring the hydrolysis of fructose 1,6-diphosphate was used. The changes in absorbance were measured with a Cary model 11 spectrophotometer at 26°.

Hydrolysis by Carboxypeptidase A and B—Carboxypeptidase A diisopropylphosphorofluoridate (crystallized three times, water suspension) and carboxypeptidase B (frozen solution) were obtained from the Worthington Biochemical Corporation. Carboxypeptidase B was treated with diisopropylphosphorofluoridate as described before (10). The carboxypeptidase digestions were carried out in 0.05 M potassium bicarbonate buffer, pH 7.8, at 25°, with enzyme-aldolase molar ratios of 1:20 and 1:50 for carboxypeptidase A and B, respectively (10). In experiments where the conditions of Drechsler, Boyer, and Kowalsky were repeated, 0.05 M potassium bicarbonate buffer, pH 7.8, containing $10^{-3}$ M EDTA was used, and the digestions were performed with a carboxypeptidase A-aldolase molar ratio of 1:1000 at 25° and 30°.

Quantitative amino acid analyses were carried out with the Beckman/Spinco amino acid analyzer according to the method reported by Moore, Spackman, and Stein (11).

Performic acid oxidation was performed by the methods of Hirs (12) at −5°.

Hydrazinolysis—the previously described modification (10) of the method of Niu and Fraenkel-Conrat (13) was used. The correction factors for alanine, glycine, serine, and tyrosine were obtained from hydrazinolysis of a known quantity of bovine serum albumin (1 mole of carboxyl-terminal alanine/69,000 g of...
protein) to which had been added known quantities of free glycine, serine, and tyrosine. The recovery values obtained in this manner were 60% for tyrosine, 52% for alanine, 45% for serine, and 35% for glycine in good agreement with the values of Xu and Fraenkel-Conrat (13).

Acid-denatured Aldolase—After dialysis for 4 hours against glass distilled water, the pH of the aldolase solution was rapidly adjusted to pH 2 with either acetic acid or hydrochloric acid. The samples were maintained at 25° for 4 hours and then lyophilized. The resulting dry protein samples were suspended in 0.05 M potassium bicarbonate buffer, pH 7.0, and treated with carboxypeptidase A under the conditions described above.

Studies on Amino Terminus—The method of Narita (14) was used in the search for acetylated amino-terminals. The experimental details have been described elsewhere (10).

Acylase Reaction—Hog kidney acylase (Sigma Chemical Company, lot 103B-2110) was used in an attempt to deacylate possible N-acetylamino-terminals in ninhydrin-negative peptides obtained from a Pronase digestion of aldolase. To 2.5 ml of substrate solutions in 0.05 M potassium phosphate buffer, pH 7.5, 0.5 mg of acylase was added. The substrate was 1 pmole of ninhydrin-negative peptide, and two controls, one containing 5 mg of N-acetylglutamic acid and one containing 5 mg of pyroglutamic acid were included. After incubation at 25° for 12 hours, the reaction was stopped by heating at 80° for 1 minute. The reaction mixtures were lyophilized and subjected to amino acid analysis and quantitative ninhydrin tests.

RESULTS

Carboxyl Terminus—When preliminary results from the treatment of aldolase with high levels of carboxypeptidase A indicated an extensive digestion beyond the expected tyrosine and alanine release, a complete time study of the carboxypeptidase digestion was carried out. The results are given in Table I and Fig. 1, and both the stoichiometry and the sequence of the amino acids released can readily be evaluated from the data. Phenylalanine and isoleucine are the only amino acids whose sequence cannot be determined directly. Since it is known, however, that carboxypeptidase A releases phenylalanine 26 times faster than it does isoleucine (15), the data are consistent with a sequence in which the slow isoleucine liberation precedes the faster phenylalanine release, thus making it appear as if the two amino acids are liberated simultaneously. No additional amino acids were released when carboxypeptidase B was included in the digestion mixture (Table II). Table II also shows the results of experiments in which the conditions of Drechsler, Bover, and Kowalsky (1) were reproduced as closely as possible. Under these conditions (low carboxypeptidase to aldolase ratio in buffer containing 10⁻³ M EDTA) only tyrosine and alanine were released, in good agreement with the reported data (1).

It is clear from the data that the action of carboxypeptidase A on aldolase essentially comes to a standstill after the first 12 hours, and it was considered of interest to determine the reason for this resistance to extensive carboxypeptidase digestion. If carboxypeptidase action were blocked by some structural feature of the polypeptide chains, then digestion of denatured aldolase should give a different pattern of amino acid release. Table III shows the results from the carboxypeptidase A digestion of acid-denatured aldolase. Since the substrate was insoluble in both experiments, the results cannot readily be subjected to quantitative interpretations, but the molar ratio of the released amino

### Table I

<table>
<thead>
<tr>
<th>Amino acids released</th>
<th>1 hr</th>
<th>1 hr</th>
<th>1 hr</th>
<th>2 hrs</th>
<th>3 hrs</th>
<th>4 hrs</th>
<th>6 hrs</th>
<th>12 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosine</td>
<td>2.65</td>
<td>2.09</td>
<td>2.74</td>
<td>2.71</td>
<td>2.87</td>
<td>2.78</td>
<td>2.99</td>
<td>3.04</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.67</td>
<td>1.18</td>
<td>1.87</td>
<td>2.19</td>
<td>2.53</td>
<td>2.63</td>
<td>3.00</td>
<td>3.02</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.38</td>
<td>0.74</td>
<td>1.29</td>
<td>1.58</td>
<td>1.78</td>
<td>1.85</td>
<td>2.00</td>
<td>1.94</td>
</tr>
<tr>
<td>Serine</td>
<td>0.10</td>
<td>0.36</td>
<td>0.92</td>
<td>1.70</td>
<td>2.42</td>
<td>2.81</td>
<td>3.34</td>
<td>3.60</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.25</td>
<td>0.58</td>
<td>0.51</td>
<td>1.00</td>
<td>1.46</td>
<td>1.68</td>
<td>2.15</td>
<td>2.30</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.00</td>
<td>Trace</td>
<td>0.26</td>
<td>0.75</td>
<td>1.15</td>
<td>1.37</td>
<td>1.74</td>
<td>2.04</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.00</td>
<td>Trace</td>
<td>0.29</td>
<td>0.78</td>
<td>1.22</td>
<td>1.42</td>
<td>1.76</td>
<td>2.03</td>
</tr>
</tbody>
</table>

* Molar weight of aldolase taken as 149,000 (4).
acids appears to be in reasonable agreement with those from native aldolase. The only significant difference in the two digestions, is the appearance of glycine in the sample denatured with hydrochloric acid.

Since it has been reported that approximately 93% of the aldolase activity was lost with the release of the three tyrosines (1), it was of interest to establish whether a further loss in activity would result from the more extensive digestion described above. The results of the activity determinations are given in Table IV, and show that the 5% residual activity remains constant throughout the digestion period.

A model of aldolase which is consistent with the carboxypeptidase results is given below.

\[-Y\text{-Ser-Phe-}Leu\text{-Leu-Ser-His-Ala-Tyr}\]
\[-Y\text{-Ser-Phe-}Leu\text{-Leu-Ser-His-Ala-Tyr}\]
\[-X\text{-Ala-Tyr}\]

This model was tested further by the following hydrazinolysis experiments.

1. Hydrazinolysis of native and performic acid oxidized aldolase should give only 3 moles of tyrosine.

2. Samples of the carboxypeptidase A-treated aldolase from Experiment 3 Table II, were subjected to hydrazinolysis (both oxidized and unoxidized samples were used). Since this sample had lost 3 moles of tyrosine and 0.14 mole of alanine, one should find only 2.86 moles of alanine if all three chains have an alanine preceding the tyrosine.

3. Aldolase, treated with carboxypeptidase A for 12 hours (Table I and Fig. 1) was also reacted with hydrazine. In this case, the predicted results according to the model should be 1 mole of X, 0.4 mole of serine, and 1.6 moles of Y.

The results of these experiments are given in Table V and are in agreement with those predicted. The data give evidence to indicate that X is glycine and Y another serine. With the high levels of alanine, serine, and glycine produced in all the hydrazinolysis experiments with aldolase, this evidence is clearly not conclusive. The tentative identification of X as a glycine residue is, however, consistent with the model, since it is known that glycine is quite resistant to carboxypeptidase A attack (15).

The appearance of glycine in the digestion of acid-denatured aldolase would also be consistent with this conclusion if one allows for additional structural hindrance to carboxypeptidase A action in the native molecule.

\section*{Amino Terminus—}The conclusion that rabbit muscle enolase contains only three amino-terminal residues is founded on good

\begin{table}[ht]
\centering
\caption{Quantitative estimation of amino acids released by hydrazinolysis of rabbit muscle aldolase and of carboxypeptidase-treated enzyme}
\begin{tabular}{lccc}
\hline
Amino acids released & Tyrosine & Alanine & Glycine \\
\hline
\text{Aldolase} & & & \\
Experiment 1 & 2.68 & 0.40 & 0.67 \\
Experiment 2 & 2.47 & 0.25 & 0.54 \\
Performic acid-oxidized aldolase & & & \\
Experiment 1 & 2.44 & 0.25 & 0.15 \\
Experiment 2 & 2.21 & 0.29 & 0.21 \\
Carboxypeptidase A-treated aldolase from Experiment 3, Table II, 12 min & & & \\
Performic acid-oxidized & 2.65 & 0.40 & 0.39 \\
Unoxidized & 2.15 & 0.35 & 0.42 \\
Carboxypeptidase A-treated aldolase from Table I, 12 hrs & & & \\
Performic acid-oxidized & 0.46 & 1.87 & 0.97 \\
Unoxidized & 0.40 & 1.08 & 0.67 \\
\hline
\end{tabular}
\end{table}

\section*{Table VI}
\caption{Amino acid estimation of ninhydrin-negative fraction from Pronase-digested aldolase}
\begin{tabular}{llc}
\hline
Amino acid & Released by hydrolysis & Released by carboxypeptidase A & Hydrazinolysis* \\
\hline
Aspartic acid & 0.35 & 0.00 & \\
Threonine & 0.10 & 0.06-0.00 & \\
Serine & 0.32 & 0.34-0.34 & 0.55 \\
Glutamic acid & 2.15 & 0.00 & 0.00 \\
Glycine & 0.05 & 0.08-0.07 & 0.012 \\
Alanine & 0.33 & 0.02-0.03 & 0.08 \\
Leucine & 0.20 & 0.16-0.19 & 0.16 \\
Tyrosine & 0.05 & 0.06-0.05 & \\
\hline
\end{tabular}

* Uncorrected values. The hydrazinolsate was dissolved directly into buffer and placed on the amino acid analyzer column without removal of hydrazides.
chemical evidence (3), and it appears that if there are six polypeptide chains in this enzyme, the additional amino-terminal residues must be blocked. Since rabbit muscle enolase was found to contain acetylated amino-terminal residues (10), it was considered important to investigate this possibility in aldolase. Although the results were negative, they are included here as they are considered relevant in deciding between a three-chain and a six-chain model for aldolase.

The ninhydrin-negative fraction from a Pronase digest of aldolase (collected from a Dowex 50 H+ column (10)) was subjected to hydrolysis, treatment with carboxypeptidase A, and hydrazinolysis, and the results given in Table VI show that a peptide (or peptides) was obtained which is rich in glutamic acid. No free N-acetylglutamic acid or pyroglutamic acid was found on paper chromatography, either before or after carboxypeptidase A treatment. Pancreatic acylase did not liberate any free amino acids from N-acetylglutamic acid but had no effect on pyroglutamic acid.

It is concluded from these results that the ninhydrin-negative fraction from the Pronase digestion of aldolase most likely is made up of a heterogeneous mixture of peptides each with an amino-terminal pyroglutamic acid. It is well known that pyroglutamic acid is produced (presumably from glutamic acid) under the conditions of the Pronase digestion and peptide fractionation (10, 16), and since the amino acid content of the peptide fraction is inconsistent with a mixture of amino-terminal peptides with overlapping sequences, we further conclude that the ninhydrin-negative peptides from aldolase are experimental artifacts. There is thus no evidence for acetylated amino-terminal residues in aldolase.

**DISCUSSION**

The results presented in this paper are in complete agreement with the three-chain model proposed for aldolase (1–6). Both the carboxypeptidase A and B digestions and hydrazinolysis gave 3 moles of carboxyl-terminal tyrosine without any evidence for other carboxyl-terminal amino acids. Similarly, the search for acetylated amino-terminal residues was negative. It is felt that these experiments represent an extensive enough attempt to uncover additional end-terminal residues and that the negative results justify the conclusion that there cannot be more than three polypeptide chains in rabbit muscle aldolase. The most reasonable explanation for the appearance of subunits with a molecular weight of 22,000 after incubation at pH 12.6 (7) is as suggested by Hass and Lewis (7) that a particularly alkali-labile peptide bond hydrolyzes at this high pH. The irreversible nature of the alkaline denaturation (7) would be consistent with this mechanism, but it is indeed remarkable that such a nonspecific process should give rise to what appear to be degradation products of very nearly equal molecular weight.

The initial experiments on the carboxyl terminus of aldolase (1, 2) led to the conclusion that one of the three chains was different from the other two in the second (alanine) residue from the carboxyl-terminal end. It appears from the present results that the difference is in the third residue instead, but that the difference indeed does exist in that two chains have a carboxyl-terminal sequence of His-Ala-Tyr while the third chain has the sequence Gly-Ala-Tyr. The identification of glycine in this sequence is based on insufficient evidence, but the complete absence of histidine in the hydrazinolysis experiments (the 12-hour carboxypeptidase sample in Table V) makes it highly unlikely that X in the proposed model can be histidine. The absence of histidine was confirmed in several quantitative amino acid analyses of a number of hydrazinolysates in order to eliminate errors due to trace amounts of free hydrazides which are eluted with the basic amino acids.

It must be emphasized here that the AAB model for aldolase (where A is a His-Ala-Tyr chain and B a Gly-Ala-Tyr chain) is based on the remarkably constant histidine-tyrosine ratio of 2:3 in several aldolase preparations. If the enzyme is crystallized as a mixture of AAA and BBB structures in a constant ratio of 2:1, one would, of course, obtain the same results.

Accepting that one of the chains differs in the third residue from the carboxyl terminus, the question arises whether this is the only different amino acid residue or whether the third chain is extensively different from the other two. As has already been mentioned, the number of peptides in peptide maps of trypsin and chymotrypsin digested aldolase is consistent with the idea of three chains (9), and it is thus possible that the substitution of glycine for histidine in one chain may be the result of a single harmless mutation and that the three chains otherwise are identical. The constant glycine-histidine ratio of 1:2 then could imply that the three chains are coded by three individual cistrons, one of which has undergone the mutation, and that aldolase perhaps is a random trimer of A and B chains, where the total A-B ratio is 2:1.

In looking for acylated amino-terminal amino acids in proteins by the present method, the appearance of glutamic acid in the ninhydrin negative peptide fractions is always a cause for consternation (10, 16). Although the present study is incomplete in this regard, it appears that the use of acylase may represent a convenient method of distinguishing between N-acetylglutamic acid and pyroglutamic acid in these cases since only the former is hydrolyzed by acylase to give free glutamic acid. On the other hand, acylase does not appear to liberate the N-acetyl group in proteins, (no free amino-terminal alanine can be demonstrated after acylase treatment of rabbit muscle enolase). Our results indicate, however, that if a short peptide containing N-acetylglutamic acid were digested with carboxypeptidase and acylase in that order, free glutamic acid should be released.

**SUMMARY**

1. Carboxypeptidase digestion and hydrazinolysis of rabbit muscle aldolase have given results which support the proposal that the enzyme contains only three chains. Negative results obtained in attempts to find acylated amino-terminal residues also support the three chain model.

2. In the presence of high concentrations of carboxypeptidase A, aldolase is digested quite extensively, and the release of amino acids is consistent with the structure

\[
Y\text{-Ser-Phe-Ileu-Leu-Ser-His-Ala-Tyr} \\
Y\text{-Ser-Phe-Ileu-Leu-Ser-His-Ala-Tyr} \\
\text{-- -- -- -- -- -- -- --} \\
X\text{-Ala-Tyr}
\]

Hydrazinolysis of the protein digestion product gave results which indicate that Y is serine and X glycine, and that one of the polypeptide chains in aldolase thus differs from the other two.

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