PRIMARY STRUCTURE OF TWO COOH-TERMINAL HEXAPEPTIDES FROM RABBIT MUSCLE ALDOLASE: A DIFFERENCE IN THE STRUCTURE OF THE α AND β SUBUNITS*

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Summary: Two COOH-terminal hexapeptides isolated from rabbit muscle aldolase have been shown to have the following primary structures: Ile-Ser-Asn-His-Ala-Tyr (α chain) and Ile-Ser-Asp-His-Ala-Tyr (β chain). This is the first structural difference identified for the two types of chains found in the crystalline aldolase preparations.

The original evidence for the presence of non-identical subunits in rabbit muscle aldolase came from experiments in Boyer's laboratory (1,2), in which three COOH-terminal tyrosine residues, but only two penultimate alanine residues, were found to be released by prolonged digestion with carboxypeptidase These observations were confirmed and extended by Winstead and Wold (3), using conditions for more vigorous digestion with carboxypeptidases A and B. Their results suggested that the chains differed in that from two of the three chains a number of amino acids, including tyrosine, alanine, and histidine, were released, while the third yielded only tyrosine and alanine.

In this laboratory the two types of subunits present in preparations of crystalline rabbit muscle aldolase have been separated by chromatography in 8 M urea, and the differences in their susceptibility to carboxypeptidase digestion has been confirmed (4). Furthermore, with denatured enzyme preparations it was found that four, rather than three, equivalents of tyrosine and alanine were released, followed by only two equivalents of histidine, and it

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was suggested that aldolase was a tetramer composed of two α and two β subunits (5). Recently, however, Rutter et al. (6) have shown preparations of crystalline rabbit muscle aldolase to be inhomogeneous, and separable by electrofocusing into five components corresponding to tetramers with a random distribution of the α and β subunits.

Although the presence of two types of peptide chains in preparations of rabbit muscle aldolase has thus been well documented, their origin and physiological significance remains obscure. Studies of the peptides obtained by CNBr treatment of the separated α and β chains have suggested that they are very similar in primary structure (7,8), if not identical. The possibility that the β chains arose by modification of the α chains was suggested by the observation that aldolase prepared from the muscles of young rabbits contained only traces of the β chains, which began to appear after the animals reached the age of three months (9).

We have now established the nature of the difference in the COOH-terminal region of the molecule which is responsible for the difference in the susceptibility of the two chains to carboxypeptidase. Two hexapeptides have been isolated from the COOH-termini of the α and β chains, respectively, and their sequence determined. The sequence of amino acids in the hexapeptide from the α chains is: Ile-Ser-Asn-His-Ala-Tyr. In the β chains asparagine is replaced by aspartic acid. The aspartyl histidine bond appears to be resistant to digestion by carboxypeptidase.

RESULTS

Isolation of the COOH-terminal peptides: Tryptic and chymotryptic digestion of the CNBr peptides containing the COOH-termini yielded the peptides used for this study. These peptides were isolated from crystalline rabbit muscle aldolase, as previously described (7), without prior separation of the α and β chains. The tryptic digests were fractionated by successive chromatography on Sephadex G-25 and Dowex-1 (Fig. 1). Four major peaks were obtained, and analysis of these with carboxypeptidase showed that peaks 3 and

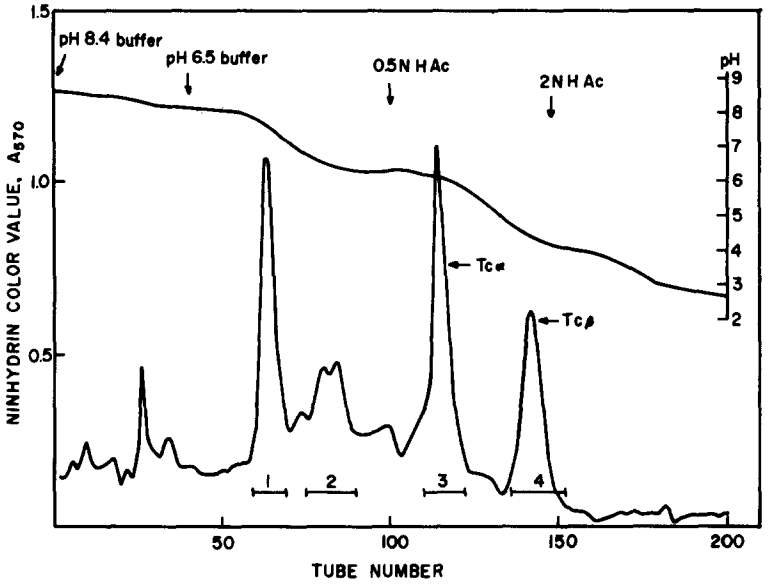

Fig. 1. Separation of COOH-terminal tryptic peptides on Dowex-1. The C-terminal segment of rabbit muscle aldolase (CnIII) was isolated as previously described (7) and digested with 1% (w/w) of TPCK-treated trypsin at pH 8.0 for 90 minutes at 30°. Preliminary separation of peptides was carried out on a Sephadex G-25 F column (2.5 x 110 cm) in 0.1 N NH_4HCO_3 and six fractions isolated on the basis of their absorbance at 280 mμ. Identification of the COOH-terminal segments was based on release of tyrosine, alanine and histidine when samples containing 20-50 nmoles of peptide were dried in a stream of air, dissolved in 50 μl of 0.1 N Tris-HCl buffer, pH 7.7, and digested with 5 μl of 0.3% carboxypeptidase A for 30 minutes at 37°. Digests were analyzed qualitatively by electrophoresis at pH 1.8 for 2 hours at 60 volts/cm, or quantitatively on a Beckman Spinco 120B amino acid analyzer using a 0.9 x 23 cm column of PA-35 resin and 0.35 N Na citrate buffer, pH 6.5. Fraction S-2, containing the COOH-terminal peptides from 60 mg of CnIII (7), was dissolved in 2 ml, adjusted to pH 11 with a drop of 2.5 N NaOH, and applied to a 0.9 x 55 cm Dowex-1 x 2 column (BioRad AG 1 x 2, 200-400 mesh). The chromatogram was developed as described by Schroeder (10). Arrows indicate changes of buffer red into the constant mixing chamber (40 ml). The flow rate was 60 ml/hr and 2.5 ml samples were collected in each fraction. Ninhydrin analyses were carried out after alkaline hydrolysis, according to Hirs (11).

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4 contained the COOH-terminal tyrosine residues. On the basis of the ratio of histidine to tyrosine residues released from these fractions (Table I), it was concluded that peak 3 was derived from the α chain and peak 4 from the β chain; these are designated as Tcα and Tcβ, respectively.

These tryptic peptides were separately digested with chymotrypsin and the digests fractionated by chromatography on Dowex-50 x 2 columns (Fig. 2).

Table I

Ratio of Histidine to Tyrosine Released by Carboxypeptidase A Digestion

Peptides	His/Tyr [a]
Segment c [b] (Cn III, (7))	0.55
S-2 [c]	0.49
Tcα [d]	0.74
Tcβ [d]	0.06

[a] The amounts of histidine and tyrosine released by carboxypeptidase A were determined as described in the legend to Figure 1. The numbers given are the molar ratios.

[b] CNBr peptide - Cn III (7).

[c] Second fraction from Sephadex G-25 chromatography (see legend to Fig. 1).

[d] COOH-terminal tryptic peptides (see Fig. 1).

Each digest yielded two peptides, and in each case the second peptides contained the COOH-terminal tyrosine residues; these were designated TcαC2 and TcβC2, respectively. Although TcαC2 and TcβC2 differed in chromatographic behavior, they were identical in amino acid composition, when hydrolyzed with acid (Table II). Each contained six amino acids, including one equivalent each of aspartic acid and serine. Following hydrolysis with aminopeptidase M, however, TcβC2 yielded the same results as acid hydrolysis, but no aspartic acid was found in the proteolytic digest of TcαC2, and the "serine" peak was twice as large, suggesting that this peptide contained asparagine instead of aspartic acid.

Sequence analysis: On exhaustive digestion with carboxypeptidase A, TcαC2 yielded all six amino acids found in the aminopeptidase M digests, but TcβC2 yielded only tyrosine and alanine, confirming the sequence of these at the COOH-terminus. The results of Edman degradation (Table III),

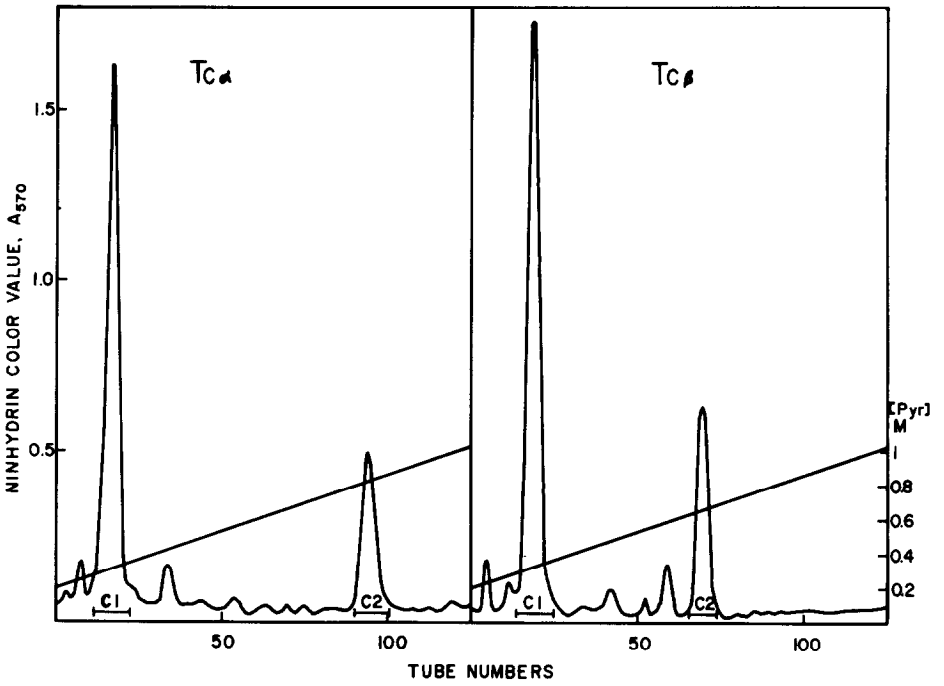

Fig. 2. Separation of chymotryptic peptides from Tcα and Tcβ on Dowex-50. Peptides Tcα and Tcβ (Fig. 1, ca. 1 μmole each) were separately dissolved in 0.6 ml of 0.1 N Tris-HCl buffer, pH 8, and treated with 5 μl of 1% α-chymotrypsin (Worthington Biochemicals) for 1.5 hours at 37°. Each digest was then brought to pH 1.8 by addition of 70% formic acid and chromatographed on 0.9 x 50 cm column of BioRad AG 50 x 2 resin, 200-400 mesh, at 40° with a linear gradient of pyridine acetate buffer, 0.2 N [Pyr^+] pH 3, to 2 N [Pyr^+] pH 5, in one liter. The flow rate was 18.5 ml/hr and 1.85 ml was collected in each tube. The peptides were detected by ninhydrin analysis on a 0.1 ml aliquot (11).

together with the results of carboxypeptidase digestion, showed the two peptides to have the following sequences:

TcαC2: Ile-Ser-Asn-His-Ala-Tyr

TcβC2: Ile-Ser-Asp-His-Ala-Tyr

DISCUSSION

In the experiments described here the starting material was the mixture of CNBr fragments containing the COOH-terminal segments of 120 amino acid residues derived from both α and β chains of rabbit muscle aldolase (7). Under the conditions employed for tryptic hydrolysis, this mixture yielded two peptides which contained both tyrosine and alanine. On chymo-

Table II

Amino Acid Compositions of the COOH-Terminal Hexapeptides Tcα2 and TcβC2

	Tcα2		Tcβ2	
	HCl [a]	AP-M [b]	HCl [a]	AP-M [b]
His	1.0	1.0	0.96	0.88
Asp	1.02	0.0	1.02	0.88
"Ser + Asn" [c]	0.95	1.82	0.92	0.88
Ala	1.0	1.02	1.0	1.0
Ile	0.94	1.07	0.94	1.0
Tyr	0.87	1.0	0.88	1.01

[a] Hydrolysis of the peptides was carried out with constant boiling HCl at 110° for 22 hours in sealed, evacuated tubes.

[b] Hydrolysis of the peptides was with aminopeptidase M (Rohm and Haas, Germany). Twenty μmoles of each peptide dissolved in 0.05 ml 0.1 N Tris-HCl buffer, pH 7.7, was digested with 1 μl of 1% aminopeptidase M for 20 hours at 37°.

[c] A compound peak of serine and asparagine and/or glutamine is eluted at the position of serine in the amino acid chromatogram (12).

tryptic digestion, each of these yielded a hexapeptide which contained a COOH-terminal tyrosine residue, confirming that these peptides were indeed the COOH-terminal fragments.

It is noteworthy that the aspartyl histidine linkage shows remarkable resistance to the action of carboxypeptidase A.[1] Under the conditions which resulted in complete hydrolysis of TcαC2 by carboxypeptidase A, TcβC2 yielded only tyrosine and alanine. This would explain the previously observed resistance of the β chain of aldolase to carboxypeptidase action (1-4), and justifies the use of the ratio of histidine to tyrosine released by carboxy-

[1] It has recently come to our attention that an aspartyl-histidine bond near the COOH-terminus of a bacterial ribonuclease is also completely resistant to digestion by carboxypeptidases A and B (R. W. Hartley, Biochem. Biophys. Res. Commun., in press).

Table III

Edman Degradation of TcαC2 and TcβC2 [a]

Degradation step	Number of residues of amino acids remaining
Tcα2	
1st	Asp, 0.97; Ser, 1.05; Ala, 1.0; Ile, 0.0; Tyr, 0.77; His, 0.96
2nd	Asp, 1.0; Ser, 0.28; Ala, 1.0; Ile, 0.0; Tyr, 0.80; His, 1.0
3rd	Asp, 0.48; Ser, 0.21; Ala, 1.0; Ile, 0.0; Tyr, 0.87; His, 1.0
4th	Asp, 0.55; Ser, 0.28; Ala, 1.0; Ile, 0.0; Tyr, 1.0; His, 0.75
TcβC2	
1st	Asp, 1.0; Ser, 0.90; Ala, 1.0; Ile, 0.0; Tyr, 0.87; His, 1.0
2nd	Asp, 1.0; Ser, 0.32; Ala, 1.0; Ile, 0.0; Tyr, 0.87; His, 1.0
3rd	Asp, 0.5; Ser, 0.32; Ala, 1.0; Ile, 0.0; Tyr, 0.85; His, 1.0
4th	Asp, 0.66; Ser, 0.4; Ala, 1.0; Ile, 0.0; Tyr, 0.94; His, 0.66

[a] Each peptide (0.2 μmole) was dissolved in 0.4 ml of pyridine-10% trimethylamine solution (6:4) and treated with 20 μl phenyl isothiocyanate at 40° for 100 minutes. After extraction of the excess reagent with benzene, the phenylthiocarbamyl derivative was heated with anhydrous trifluoroacetic acid for 30 minutes at 40° to effect the cleavage of the NH_2-terminal amino acid residue. After extraction of the phenylthiazolinone with ether, an aliquot corresponding to 10% of the remaining peptide was hydrolyzed with acid and analyzed on the amino acid analyzer.

peptidase digestion (9) for the identification of α and β chains.

The present results also explain the apparent rapid release of "serine" in the later stage of carboxypeptidase digestion of the α chains of aldolase (3,4,7). Serine, asparagine and glutamine appear as a slightly asymmetric single peak in the conventional amino acid chromatogram (12), and cannot be distinguished without resort to other means.

We have previously reported evidence indicating that the structural differences between α and β chains of rabbit muscle aldolase might be minor and confined to a region near the COOH-termini (7,9). The results obtained here present the first evidence for a structural difference in the two chains. Studies in progress on the tryptic COOH-terminal peptides, Tcα and Tcβ, containing 22 amino acids, appear to show no additional differences. Isolation

of similar COOH-terminal tryptic peptides has been reported, but no structural studies were presented (13).

The age-dependent change in the ratio of α to β subunits in rabbit muscle aldolase (9) may be due to deamidation of the asparagine residue near the COOH-terminus, in which case we would conclude that only a single gene (for α chains) is involved in the biosynthesis of rabbit aldolase. Subsequent random deamidation *in vivo* would produce a random mixture of electrophoretic forms, such as has been described by Rutter *et al.* (6). Studies to establish the basis for this selective deamidation are now in progress.

REFERENCES

1. Drechsler, E. R., Boyer, P. D. and Kowalsky, A., J. Biol. Chem. 234, 2627 (1959).
2. Kowalsky, A. and Boyer, P. D., J. Biol. Chem. 235, 604 (1960).
3. Winstead, J. A. and Wold, F., J. Biol. Chem. 239, 4212 (1964).
4. Chan, W., Morse, D. E. and Horecker, B. L., Proc. Natl. Acad. Sci. 57, 1013 (1967).
5. Morse, D. E., Chan, W. and Horecker, B. L., Proc. Natl. Acad. Sci. 58, 628 (1967).
6. Rutter, W. J., Penhoet, I. and Kochman, M., Federation Proc. 27, 590 (1968).
7. Lai, C. Y., Arch. Biochem. Biophys. 128, 202 (1968).
8. Lai, C. Y. and Chen, C., Arch. Biochem. Biophys. 128, 212 (1968).
9. Koida, M., Lai, C. Y. and Horecker, B. L., Arch. Biochem. Biophys. 134, 623 (1969).
10. Schroeder, W., "Methods in Enzymology", (C. H. W. Hirs, ed.), Vol. 9, Academic Press, New York, p. 361, 1967.
11. Hirs, C. H. W., "Methods in Enzymology", (C. H. W. Hirs, ed.), Vol. 9, Academic Press, New York, p. 325, 1967.
12. Spackman, D. H., Stein, W. H. and Moore, S., Anal. Chem. 30, 1190 (1958).
13. Wassarman, P. M. and Kaplan, N. O., J. Biol. Chem. 243, 720 (1968).