# PIGEON MUSCLE ALDOLASE: KINETIC PROPERTIES AND PRIMARY STRUCTURE CLOSE TO THE SUBSTRATE BINDING LYSINE RESIDUE

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## 1. Introduction

A considerable study has been made of the primary structure and specificity of fructose 1,6-diphosphate aldolase (EC 4.1.2.13) from a variety of vertebrate species [1–6]. Impressive sequence homologies around the active site lysine residue in aldolases from rabbit, ox, pig, sturgeon, cod and frog muscle, and from rabbit and ox liver, have been described [2–4, 6]. These structural homologies together with similarities in specificity constitute evidence for the derivation of these enzymes from a common ancestral form. The present investigation was undertaken to extend these studies to include an avian muscle aldolase.

## 2. Materials and methods

Aldolase was isolated from pigeon breast muscle, together with several other enzymes, using a procedure the full details of which will be published elsewhere. Rabbit muscle aldolase was purchased from C.F. Boehringer und Soehne (Mannheim, Germany). Aldolase activity was estimated using the coupled assay described by Blostein and Rutter [7]. A specific extinction coefficient $E^{1\%}_{280} = 9.1$ was assumed for both the rabbit and pigeon enzymes [8]. Both enzymes were S-carboxymethylated with iodo [2-$^{14}$C]acetic acid in 8 M-urea according to the method of Anderson et al. [1]. Cyanogen bromide cleavage, peptide mapping and autoradiography were carried out as previously described [9, 10]. Protein was digested with 1% trypsin (w/w) in 0.5% $NH_4HCO_3$ (w/w), pH 8.0, for 4 hr at 37° or with 1% carboxypeptidase A (w/w) in 50 mM N-ethylmorpholine acetate pH 8.25 at 22°.

The peptides produced by cyanogen bromide cleavage were rendered soluble by reaction with citraconic anhydride [11] prior to separation on Sephadex G-75 in 0.5% $NH_4HCO_3$, pH 8.0 [2, 6]. The method described by Gray and Hartley [12] was employed for the dansyl-Edman degradation of peptides. Amide assignments were made from the electrophoretic mobility (m) at pH 6.5 defining the mobility of aspartic acid as −1.0 [13].

Electrophoresis in polyacrylamide gels was carried out at pH 8.9 in 3.5% gels containing 8 M urea according to Davis [14], or in the presence of 0.1% sodium dodecyl sulphate in 7.5% gels [15].

## 3. Results

After electrophoresis in acrylamide gels containing 8 M urea the rabbit enzyme appeared as two protein bands [1]. The pigeon enzyme appeared as three bands, two minor components and a major band of intermediate mobility. The two components of the pigeon aldolase with the lowest anodal mobility coincided with the components of the rabbit enzyme. The appearance of two electrophoretic species in rabbit muscle aldolase after electrophoresis in 8 M urea is well known [1, 16], and is considered to be due to the presence of two types of chain in aldolase preparations. One of these chain types is thought to derive from the other by the loss of a single amide group [17]. A similar explanation may account for the multiple bands observed after electrophoresis of pigeon muscle aldolase. Polyacrylamide gel electrophoresis of the native and carboxymethylated protein

Table 1

Amino acid compositions of skeletal muscle aldolases from pigeon and rabbit based on subunit molecular weight of 40,000.

| Residue | Pigeon (mole/mole protein) | Rabbit |
|---|---|---|
| Lys | 23.9 | 26.6 |
| His | 8.3 | 10.1 |
| Arg | 18.7 | 14.9 |
| Cmc* | 7.2 | 7.8 |
| Asp | 29.7 | 28.7 |
| Thr | 23.0 | 21.4 |
| Ser | 18.7 | 20.3 |
| Glu | 40.3 | 40.9 |
| Pro | 18.5 | 18.6 |
| Gly | 30.3 | 29.6 |
| Ala | 46.4 | 40.5 |
| Val | 23.6 | 21.1 |
| Met | 5.0 | 3.3 |
| Ile | 16.2 | 19.5 |
| Leu | 34.9 | 33.0 |
| Tyr | 11.4 | 10.9 |
| Phe | 7.6 | 7.3 |
| Trp | 3.9 | 3.7 |

Corrections which compensate for the destruction of threonine and serine on hydrolysis and the incomplete release of valine and isoleucine have been made [1]. The tryptophan content was determined by the method of Bencze and Schmid [19].

* Abbreviations: Cmc, carboxymethylcysteine; Hsr, homoserine.

in the presence of sodium dodecyl sulphate resulted in a single protein band. The mobility of this band relative to that of marker proteins (bovine serum albumin, rabbit glyceraldehyde 3-phosphate dehydrogenase and hen egg white lysozyme) corresponded to a subunit molecular weight of 40,000 which is in good agreement with the subunit molecular weight of the rabbit muscle enzyme [18]. The native pigeon and rabbit muscle enzymes are reported to have identical sedimentation coefficients [5]; it seems likely therefore that the quaternary structure of the pigeon enzyme resembles that of rabbit muscle aldolase which is a tetrameric molecule of molecular weight 160,000.

The amino acid composition of the carboxymethylated pigeon enzyme, based on a subunit molecular weight of 40,000, together with the known composition of rabbit muscle aldolase is presented in table 1. Although the overall composition of the

Table 2

The kinetic properties of pigeon, sturgeon and rabbit muscle aldolase.

| | Rabbit | Pigeon | Sturgeon (*A. fulvescens*) |
|---|---|---|---|
| FDP $K_m$ (M) | $1\times10^{-6}$ | $0.5\times10^{-6}$ | $0.8\times10^{-6}$ |
| F1P $K_m$ (M) | $1.3\times10^{-3}$ | $1.1\times10^{-3}$ | $0.9\times10^{-3}$ |
| $V_{max}$ (units/mg) | 10–15 | 7–15 | 8–15 |
| FDP:F1P $V_{max}$ ratio | 26 | 20 | 13 |
| FDP–F1P $V_{max}$ ratio after carboxypeptidase | 1 | 1 | 1 |

The kinetic data for the sturgeon enzyme were obtained by Anderson [24] and Gibbons (unpublished work).

pigeon enzyme is similar to that of the rabbit enzyme and to those of other vertebrate aldolases reported by Anderson et al. [1] there are some differences.

Tryptic peptide 'maps' were prepared from both the rabbit and pigeon carboxymethylated proteins and from a mixture of the two. Samples of tryptic digests were electrophoresed at pH 6.5 in one dimension and then chromatographed at right angles in the system of Waley and Watson [20]. The band of "neutral" peptides was subjected to a further dimension of electrophoresis at pH 3.5. Comparison of these 'maps' after staining with ninhydrin/cadmium acetate reagent [21] suggested that at least half of the peptides from the pigeon enzyme coincided with peptides from the rabbit enzyme. Furthermore, autoradiography of the tryptic peptide 'maps' revealed that four of the carboxymethylcysteine-containing peptides from the two enzymes coincided. Thus it seems likely that at least half the peptides of the pigeon enzyme are identical or closely homologous to peptides of the rabbit enzyme.

The products obtained after treating the S-carboxymethylated pigeon aldolase with cyanogen bromide were citraconylated and chromatographed on Sephadex G-75. The elution profile was rather

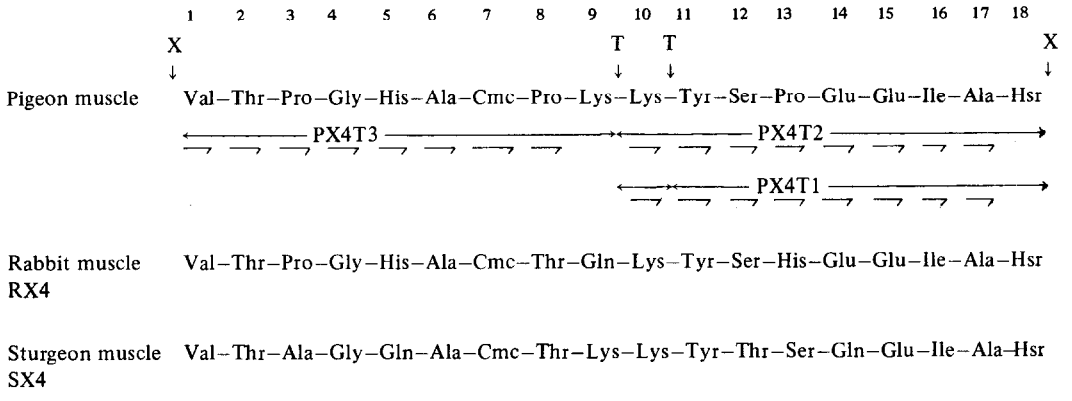


Fig. 1. The amino acid sequences of peptides PX4, RX4 and SX4 from pigeon, rabbit and sturgeon (*A. fulvescens*) muscle aldolases. X and T indicate positions of cleavage by cyanogen bromide and trypsin, respectively. The C-terminal hormone residue is produced from a methionine residue on cyanogen bromide digestion.

different from that obtained after gel filtration of the rabbit enzyme digest [1] as was expected from the difference in methionine content (rabbit aldolase has 3 and pigeon aldolase has 5 methionine residues per subunit). However, one particular fragment, PX4, of 18 residues was readily recognised and identified with a rabbit peptide RX4 by its relatively late elution position. The peptide X4 is known to lie close to the substrate binding lysine residue of the active site of aldolase and its sequence for the rabbit and sturgeon muscle enzymes has already been reported [2]. The pigeon peptide PX4 (m=0.0) had the following composition:
Lys 2, His 1, Cmc 1, Thr 1, Ser 1, Glx 2, Ala 2, Val 1, Ile 1, Tyr 1, Hsr 1,.

The N-terminal residue was established as Val after dansylation. A tryptic digest of the PX4 fragment gave free lysine and the following peptides:

PX4T1 Ser 1, Glx 2, Pro 1, Ala 1, Ile 1, Tyr 1, Hsr 1,. (m = –0.55)

PX4T2 Lys 1, Ser 1, Glx 2, Pro 1, Ala 1, Ile 1, Tyr 1, Hsr 1,. (m = –0.23)

PX4T3 Lys 1, His 1, Cmc 1, Thr 1, Pro 2, Glx 1, Ala 1, Val 1,. (m = +0.25)

The sequences of these peptides were established by dansyl-Edman degradation and together account for the sequence of PX4 as shown in fig. 1. The sequences of the rabbit peptide RX4 and the homologous sturgeon peptide SX4 are also shown.

The specificities of rabbit, sturgeon and pigeon muscle aldolases towards fructose-1,6-diphosphate (FDP) and fructose-1-phosphate (F1P) were compared. The catalytic properties of these aldolases appear to be very similar (table 2). The $K_m$ values of the different aldolases for FDP or F1P are almost identical as are the values for $V_{max}$ using FDP as substrate. The ratio of activities with FDP and F1P are all of the same order although the value obtained with the sturgeon muscle enzyme is relatively low. It is interesting to note that rabbit liver aldolase cleaves both substrates at the same rate [22]. The high activity of the muscle enzymes towards FDP is thought to be associated with an intact C-terminal sequence of the polypeptide chain. Removal of the C-terminal residues from the rabbit and sturgeon muscle aldolases, using carboxypeptidase A, reduces the rate of cleavage of FDP while the rate of cleavage of F1P and the $K_m$ values for both substrates remain essentially unchanged [23, 24]. Treatment of pigeon muscle aldolase with carboxypeptidase produces a similar change in specificity. After digestion for 5 min the specific activity towards FDP was reduced to 4.8% of the original activity so that the modified enzyme cleaves both substrates at approximately the same rate. No further decrease in activity was observed after longer periods of digestion.

After digestion with carboxypeptidase for 45 min the following amino acids were found in approximately molar yields:

pigeon: His, Ser, Ala, Val, Leu, Tyr, Phe,
rabbit : His, Ser, Ala, Ile, Leu, Tyr, Phe,

These residues were in equivalent amounts for the two species. These results indicate that the C-terminal sequences of the two enzymes are very similar except for the appearance of valine in the pigeon sequence in place of isoleucine in the rabbit sequence. The C-terminal sequence of the rabbit enzyme has already been established and is complicated by the existence of two types of aldolase chain which differ by one residue in their C-terminal sequence. Thus, a more detailed study is required to determine the exact order of the C-terminal residues of pigeon muscle aldolase. However, it seems likely by homology with the rabbit and sturgeon enzymes that the order of the final three residues is –His–Ala–Tyr.

## 4. Discussion

The kinetic studies reported here and those of other authors [5, 24] have demonstrated a close functional similarity amongst muscle aldolases from widely differing sources.

A considerable structural homology between pigeon and rabbit muscle aldolase is suggested by the number of peptides common to the two enzymes. The extent of this homology is less than that between mammalian aldolases but greater than that between mammalian and sturgeon aldolase [1, 2], as might be expected from the phylogenetic relationship of these species. These interspecies relationships are mirrored in the homology between X4 peptides, there being 3 substitutions between the rabbit and the pigeon sequences and 6 between either the rabbit or pigeon and the sturgeon sequence. All the substitutions observed can be accounted for by single base changes in the *E. coli* genetic code [25].

The X4 cyanogen bromide peptide has been readily identified and isolated from several class 1 aldolases and constitutes a representative sample of class 1 aldolase sequence. It may therefore be possible to obtain a clear idea of the extent of homology between class 1 aldolase by structural analysis of the X4 peptides. This approach would be most useful in cases where the extent of homology is low (less than about 70%) since in these circumstances comparative 'fingerprinting' gives little information about the degree of homology.

Considerable structural variations are found in the X4 peptides without significant change in the catalytic properties of aldolase. Although this finding implies that this region of the molecule is functionally unimportant it should be noted that several residues within X4 are invariant (residues 1, 2, 4, 7, 10, 11, 15, 17, 18) in all the sequences studied so far [1–4, 6].

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