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Extended Amino Acid Sequences around the Active-Site Lysine Residue of Class-I Fructose 1,6-Bisphosphate Aldolases from Rabbit Muscle, Sturgeon Muscle, Trout Muscle and Ox Liver

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1. Amino acid sequences covering the region between residues 173 and 248 [adopting the numbering system proposed by Lai, Nakai & Chang (1974) *Science* **183**, 1204–1206] were derived for trout (*Salmo trutta*) muscle aldolase and for ox liver aldolase. A comparable sequence was derived for residues 180–248 of sturgeon (*Acipenser transmontanus*) muscle aldolase. The close homology with the rabbit muscle enzyme was used to align the peptides of the other aldolases from which the sequences were derived. The results also allowed a partial sequence for the *N*-terminal 39 residues for the ox liver enzyme to be deduced. 2. In the light of the strong homology evinced for these enzymes, a re-investigation of the amino acid sequence of rabbit muscle aldolase between residues 181 and 185 was undertaken. This indicated the presence of a hitherto unsuspected -Ile-Val- sequence between residues 181 and 182 and the need to invert the sequence -Glu-Val- to -Val-Glx- at positions 184 and 185. 3. Comparison of the available amino acid sequences of these enzymes suggested an early evolutionary divergence of the genes for muscle and liver aldolases. It was also consistent with other evidence that the central region of the primary structure of these enzymes (which includes the active-site lysine-227) forms part of a conserved folding domain in the protein subunit. 4. Detailed evidence for the amino acid sequences proposed has been deposited as Supplementary Publication SUP 50098 (23 pages) at the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in *Biochem. J.* (1978) **169**, 5.

Fructose 1,6-bisphosphate aldolase (EC 4.1.2.13) is the glycolytic enzyme responsible for the reversible aldol cleavage of fructose 1,6-bisphosphate to the two triose phosphates, glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. The reaction catalysed proceeds via a carbanion intermediate, and aldolases fall into two classes that differ primarily in the type of electron sink used to stabilize this intermediate (Rutter, 1964). In aldolases of class I, a protonated ketimine formed between the substrate C-2 carbonyl group and the 6-amino group of an active-site lysine residue is thought to have this function (Horecker *et al.*, 1963; reviewed by Horecker *et al.*, 1972; Di Iasio *et al.*, 1977). In aldolases of class II, the corresponding electron sink is probably a bivalent transition-metal ion, and no covalent enzyme–substrate intermediate is formed (Rutter, 1964; Mildvan *et al.*, 1971).

The class-I aldolases were once thought to be confined to eukaryotes (Rutter, 1964), but have since been detected in several bacterial species (Lebherz & Rutter, 1973; Stribling & Perham, 1973; London, 1974; Baldwin & Perham, 1978). The typical class-I aldolases of animals and higher plants have been widely studied (for a review see Horecker *et al.*, 1972). Such enzymes are invariably tetrameric, and amino acid-sequence studies indicate that they are probably derived by divergent evolution from a common ancestral protein. In mammals, class-I aldolases exist as three tissue-specific enzymes: aldolase A in muscle, aldolase B in liver, and aldolase C together with aldolase A in brain. Rabbit muscle aldolase contains two different types of subunit, designated α and β, each of relative molecular mass 40000. The α- and β-subunits appear to differ only in the state of amidation of an aspartic acid residue close to the *C*-terminus of the polypeptide chain (Lai *et al.*, 1970), deamidation of the relevant asparagine side chain occurring progressively and naturally *in vivo* (Midelfort &

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Mehler, 1972). The presence of α- and β-subunits has been demonstrated in aldolases from other mammalian sources, e.g. pig and ox muscle (Anderson *et al.*, 1969), but the relevant asparagine residue is absent from rabbit liver aldolase (Lacko *et al.*, 1970).

The complete amino acid sequence of the rabbit muscle enzyme has been independently determined in two laboratories (Lai *et al.*, 1974; Sajgo & Hajos, 1974). Unfortunately the results are not in complete agreement. X-ray-crystallographic analysis of the protein has also been undertaken (Eagles *et al.*, 1969; Heidner *et al.*, 1971), but thus far a model with resolution sufficient to trace the polypeptide chain is not available. The amino acid sequences of class-I aldolases from muscle and liver of a wide range of eukaryotes are closely homologous (see, e.g., Anderson *et al.*, 1969; Horecker *et al.*, 1972), which suggests that a model established by X-ray crystallography for one enzyme will allow a reliable model for another enzyme to be built from a knowledge of its sequence alone (Forcina & Perham, 1971).

In the present paper we describe the derivation of amino acid sequences covering the region between residues 173 and 248 [adopting the numbering system proposed by Lai *et al.* (1974)] for muscle aldolases from sturgeon (*Acipenser transmontanus*) and trout (*Salmo trutta*) and for liver aldolase from the ox. The work was undertaken as part of a study of the evolution of the bony fish (Osteichthyes) (P. A. Benfield, K. Joysey & R. N. Perham, unpublished work) and to extend an earlier study of the relatedness of muscle and liver aldolases (Forcina & Perham, 1971). This particular region of the protein is of further interest in that it includes the active-site lysine residue (position 227; Lai *et al.*, 1974) and has been implicated as part of a characteristic folding feature, presumably a folding domain, of the three-dimensional structure (Stellwagen, 1976; Lambert *et al.*, 1977). Our results prompted us to investigate the amino acid sequence of residues 181–185 reported for the rabbit muscle enzyme (Lai *et al.*, 1974; Sajgo & Hajos, 1974) and allow us to comment on some of the discrepancies between the two published sequences for this enzyme.

Material and Methods

Enzymes and reagents

Rabbit muscle aldolase was purchased from C. F. Boehringer und Soehne, Mannheim, West Germany. Aldolase was prepared from the muscle of the North American sturgeon (*Acipenser transmontanus*) (Gibbons *et al.*, 1972) and from ox liver (Forcina & Perham, 1971) by using the method of Penhoet *et al.* (1969). The same method was also used to prepare aldolase from the muscle of a single specimen of the brown trout (*Salmo trutta*) provided by the Anglian Water Authority, except that the substrate elution step was carried out with 2.5mM-fructose 1,6-bisphosphate dissolved in 100mM-Tris/HCl/10mM-EDTA buffer, pH7.5. At lower buffer concentrations addition of substrate failed to elute the aldolase. Trypsin and chymotrypsin (thrice recrystallized; salt-free) were purchased from Worthington Biochemical Corp., Freehold, NJ, U.S.A., subtilisin was from BDH Chemicals, Poole, Dorset, U.K., and thermolysin (thrice recrystallized) was from Calbiochem, London W.1, U.K.

Iodoacetic acid was obtained from Hopkin and Williams, Chadwell Heath, Essex, U.K., and recrystallized twice from n-heptane before use. Iodo[2-^{14}C]acetic acid was purchased from The Radiochemical Centre, Amersham, Bucks., U.K., and diluted with carrier iodoacetic acid to a specific radioactivity of 1.5mCi/mmol before use. 2-Mercaptoethanol and CNBr were obtained from Eastman–Kodak, Kirkby, Lancs., U.K., and fluorescamine (Fluram; 4-phenylspirofuran-2(3),1′-phthalan-3,3-dione) from Hoffman–LaRoche, Basel, Switzerland. Reagents for the dansyl–Edman degradation of peptides were purchased from Pierce and Warriner (U.K.), Chester, Cheshire, U.K. All other reagents were of analytical grade.

S-Carboxymethylation of proteins

Proteins were *S*-carboxymethylated in 0.1M-Tris/HCl buffer, pH8.5, in the presence of 8M-urea and 2mM-dithiothreitol, as described by Gibbons & Perham (1970).

Polyacrylamide-gel electrophoresis

Samples of protein were run in 7.5% (w/v) polyacrylamide gels containing 0.1% sodium dodecyl sulphate (Shapiro & Maizel, 1969). Molecular weights were estimated from the electrophoretic mobilities as described by Perham & Thomas (1971).

Cleavage of polypeptide chains

CNBr cleavage was carried out in 70% (v/v) formic acid for 24h at 20°C, and the resulting peptides were treated with citraconic anhydride (Dixon & Perham, 1968; Gibbons & Perham, 1970) to promote solubility of the large fragments (Gibbons *et al.*, 1970; Forcina & Perham, 1971). Peptides were decitraconylated by incubating them overnight with 1.5% (v/v) formic acid at 20°C. Digestions of peptides and proteins with proteolytic enzymes were carried out for 4h at 37°C in 0.5% NH_4HCO_3, pH8.0, with enzyme/substrate ratios of 1:100 (w/w). A temperature of 55°C was used for digestions with thermolysin.

Radioactivity measurements

Samples were added to 3ml of toluene/Triton (2:1, v/v) containing 2,5-diphenyloxazole (0.5%,

w/v) and their radioactivities counted in a Nuclear-Chicago Unilux IIA scintillation counter.

Separation and characterization of peptides

Paper electrophoresis, chromatography, gel filtration on Sephadex and dansyl–Edman degradation of peptides were carried out as described previously (Gibbons *et al.*, 1970; Forcina & Perham, 1971). Peptide 'maps' and radioautographs were prepared by the method of Harris & Perham (1965), and amide assignments were made from the electrophoretic mobility (*m*) of the peptide at 6.5 (Offord, 1966), with the mobility of aspartic acid being defined as −1.00.

Amino acid analysis

Samples of peptides and proteins were hydrolysed for 24h (unless stated otherwise in the text) at 105°C with 6M-HCl containing 0.1% 2-mercaptoethanol. The amino acids were analysed on a Beckman 120C automatic analyser or, in the later stages of this work, on a Rank Hilger Chromaspek (mark I) amino acid analyser fitted with a Digico Micro 16V computer.

Results

General characterization of the enzymes

All the aldolases gave identical single bands on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, the mobilities of which were consistent with the commonly accepted value of 40000 for the relative molecular mass of the polypeptide chain.

The catalytic activity of the trout muscle aldolase was never higher than 75nkat/mg, when assayed at 30°C by the method of Blostein & Rutter (1963), whereas the catalytic activities of the other muscle enzymes were in the range 217–284nkat/mg. The trout muscle enzyme also showed some instability during purification, and it is possible that the protein is slightly degraded at the *C*-terminus, as indicated by a study of its *C*-terminal amino acid sequence (P. A. Benfield, unpublished work).

Amino acid analyses for the ox liver and trout muscle aldolases are given in Table 1, based in each case on a subunit relative molecular mass of 40000. Comparable analyses of the sturgeon and rabbit muscle enzymes have been published elsewhere (Anderson *et al.*, 1969).

Amino acid-sequence studies on trout muscle aldolase

A sample (86mg) of trout muscle aldolase was reduced and *S*-carboxymethylated under denaturing conditions with iodo[2-^{14}C]acetic acid and then treated with CNBr to cleave the polypeptide chain at methionine residues. The peptide fragments were citraconylated and gel-filtered on a column (2cm × 114cm) of Sephadex G-75 (superfine grade) in 0.5% (w/v) NH_4HCO_3. The effluent was monitored for absorbance at 280nm and for radioactivity, as a result of which six successive cuts, A–F, were made and the peptides freeze-dried. Full details of these operations are given in Supplementary Publication SUP 50098. Peak F was decitraconylated and its two constituent peptides (TXF1 and TXF2) were purified by paper electrophoresis at pH6.5 and 3.5. Peptides TXF1 (*N*-terminus tyrosine) and TXF2 (*N*-terminus valine) were digested further with trypsin, and the tryptic peptides were purified by paper electrophoresis at pH6.5 and 3.5. The amino acid compositions of these peptides were determined and their sequences partly established by dansyl–Edman degradation. The peptides were found to correspond to residues 211–248 of the rabbit muscle aldolase sequence reported by Lai *et al.* (1974). Homology was therefore used to align the tryptic peptides as shown in Fig. 1. The amino acid sequence of peptide TXF1T1 (from residues 215–230) is assumed from the identity of its composition with that of the corresponding peptide from sturgeon muscle aldolase, whose sequence

Table 1. *Amino acid compositions of aldolases from trout muscle and ox liver*

For each enzyme, duplicate samples of protein were hydrolysed for 24, 48 and 72h. The results quoted are the mean values, to the nearest whole number of residues. The values for serine and threonine are corrected to allow for destruction during acid hydrolysis, and the values for valine and isoleucine are corrected to allow for the slow release of these amino acids during hydrolysis. N.D., Not determined.

	Amino acid composition (mol/mol of protein subunit, M_r 40000)	
Amino acid	Trout muscle aldolase	Ox liver aldolase
Lysine	23	30
Histidine	11	11
Arginine	17	15
Cysteine*	8	8
Aspartic acid	28	31
Threonine	23	21
Serine	21	21
Glutamic acid	35	44
Proline	21	15
Glycine	30	30
Alanine	42	42
Valine	23	25
Methionine	5	7
Isoleucine	17	16
Leucine	33	36
Tyrosine	11	10
Phenylalanine	12	10
Tryptophan†	N.D.	N.D.

* Estimated as *S*-carboxymethylcysteine.

† About 3mol/mol was present in the acid hydrolysate, estimated on the amino acid analyser.

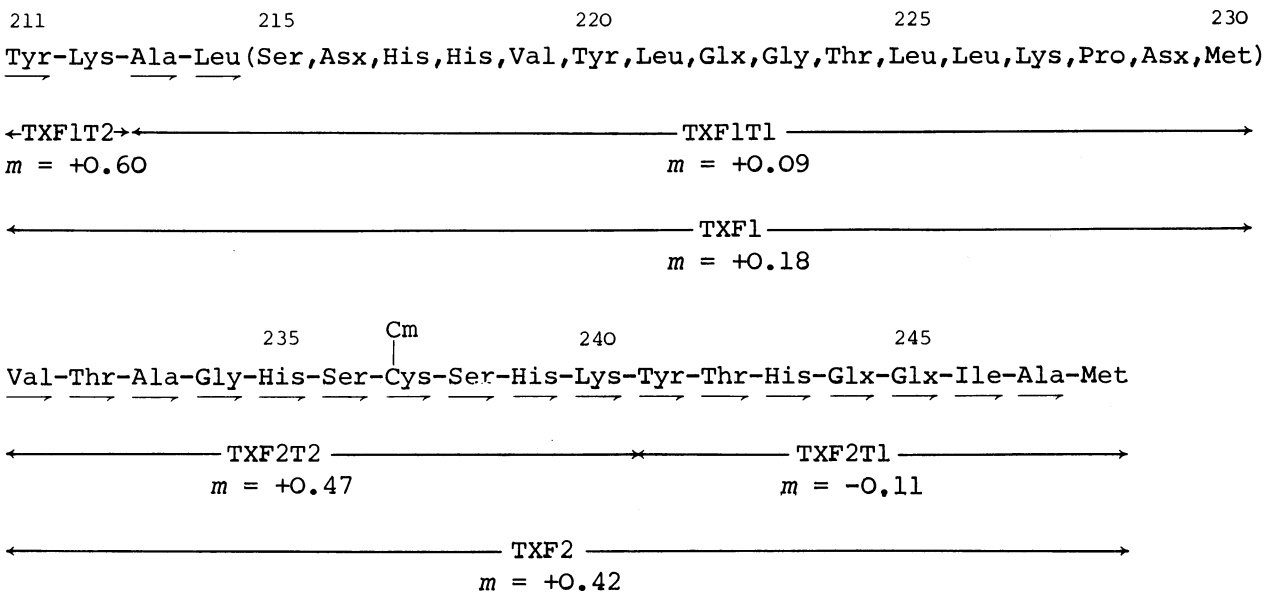

Fig. 1. *Derivation of the amino acid sequence of residues* 211–248 *of trout muscle aldolase* [*numbered according to the rabbit muscle enzyme sequence given by Lai et al.* (1974)]

The sequence of residues 215–230 is assumed because of the identity of its composition with the corresponding sequence of sturgeon muscle aldolase (Gibbons *et al.*, 1972). → denotes a residue established by dansyl–Edman degradation. Residues 230 and 248 are shown as Met, but in the actual peptides studied both residues were present as the CNBr-cleavage product homoserine. In this and subsequent Figures Cm represents carboxymethyl.

was determined by Gibbons *et al.* (1972). The amino acid sequence of peptide TXF2 was established unequivocally from the sequences of its component tryptic peptides TXF2T1 and TXF2T2, as shown in Fig. 1.

Peptides derived from residues 173–210 of trout muscle aldolase were found in the tryptic digest of peak-D material from CNBr cleavage, isolated as described above. However, it proved more convenient for sequencing purposes to purify these peptides directly from a tryptic digest of the *S*-carboxy[^{14}C]-methylated protein. A sample of the tryptic digest (30mg) was gel-filtered on a column (2cm×114cm) of Sephadex G-50 (superfine grade) in 0.5% NH_4HCO_3. The effluent was monitored for absorbance at 280nm and for radioactivity, and cut accordingly into ten successive portions from each of which the peptides were recovered by freeze-drying. Full details of these operations are given in Supplementary Publication SUP 50098. The peptides were fractionated by paper electrophoresis and paper chromatography and their amino acid sequences were investigated by dansyl–Edman degradation. The long tryptic peptide TT3*a* was found to have the *N*-terminal sequence Tyr-Ala-Ser-Ile-CmCys-Glx-. It was digested further with thermolysin, and the thermolytic fragments (TT3*a*H1, TT3*a*H2 etc.) were separated by paper electrophoresis and chromatography. The amino acid sequences of these fragments were completely established by dansyl–Edman degradation. From this information it was a simple matter to select those peptides whose sequences were closely homologous with the region spanned by residues 173–210 of the rabbit muscle aldolase

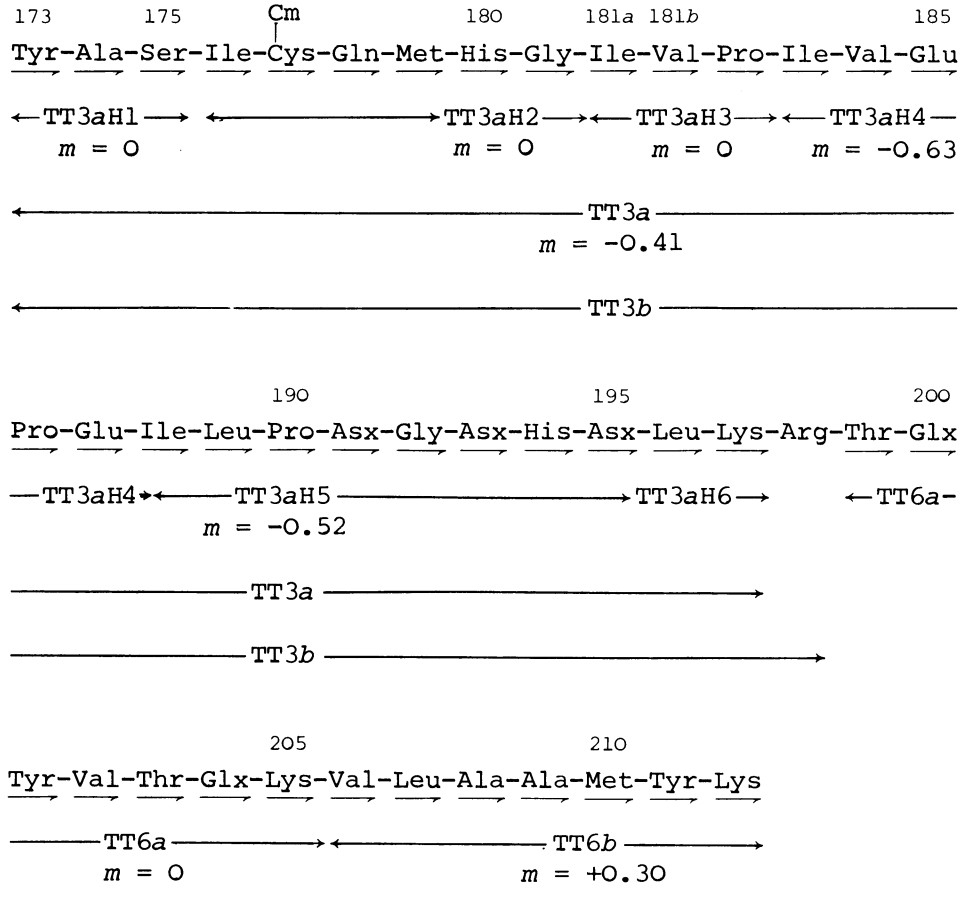

Fig. 2. *Derivation of the amino acid sequence of residues* 173–212 *of trout muscle aldolase* [*numbered according to the rabbit muscle enzyme sequence given by Lai et al.* (1974)]

The ordering of peptides is based on the close sequence homology with the rabbit muscle enzyme. → denotes a residue established by dansyl–Edman degradation.

reported by Lai *et al.* (1974). This homology could be used to align the tryptic and thermolytic peptides of trout muscle aldolase as shown in Fig. 2.

The sequence -Met-Tyr-Lys in peptide TT6*b*, assigned the numbering 210–212 in the protein in Fig. 2, is nicely consistent with CNBr cleavage at methionine-210 implied by the isolation of peptide TXF1 and with the *N*-terminal sequence Tyr-Lys- established for that peptide (Fig. 1). On the other hand, it proved necessary to propose an insertion of an -Ile-Val- sequence at positions 181*a* and 181*b* (Fig. 2) to maintain the otherwise close sequence homology with the rabbit muscle enzyme.

Taken together, the results summarized in Figs. 1 and 2 enable us to propose an amino acid sequence (with certain amide assignments left unresolved) for the region spanning residues 173–248, as shown in Fig. 8. Although the ordering of the peptides rests on sequence homology with the rabbit muscle enzyme, the amino acid compositions of the peptides (parent and progeny) are wholly consistent with the formulated sequence and strongly imply that no peptides have been overlooked.

Amino acid-sequence studies on ox liver aldolase

A sample (80mg) of ox liver aldolase was *S*-carboxymethylated with iodo[2-^{14}C]acetic acid under denaturing conditions and then cleaved by treatment with CNBr. The resulting peptide fragments were citraconylated and gel-filtered on a column (140cm×2.5cm) of Sephadex G-75 in 0.5% NH_4HCO_3. The effluent was monitored for absorbance at 280nm and for radioactivity. The profile has been recorded previously (Forcina & Perham, 1971) and comprises five peaks, OXA–OXE, in order of elution from the column. We have already shown (Forcina & Perham, 1971) that peak OXD on tryptic digestion yields a peptide that contains the substrate-binding lysine residue of ox liver aldolase, corresponding to lysine-227 of the homologous rabbit muscle enzyme (Lai *et al.*, 1974).

Peak-D material was decitraconylated and found to yield only one *N*-terminal residue, glutamic acid, on dansylation. It was digested with trypsin and the peptides were separated by paper electrophoresis at pH6.5 and 3.5. The amino acid compositions of three of these peptides (OXDT1, OXDT2 and OXDT3) were found to correspond to residues 197–230 of the rabbit muscle enzyme sequence given by Lai *et al.* (1974). The amino acid sequence of peptide OXDT3 is taken from Forcina & Perham (1971). The amino acid sequence of peptide OXDT2 was completely established by dansyl–Edman degradation. Peptide OXDT1 (*N*-terminus glutamic acid) was digested

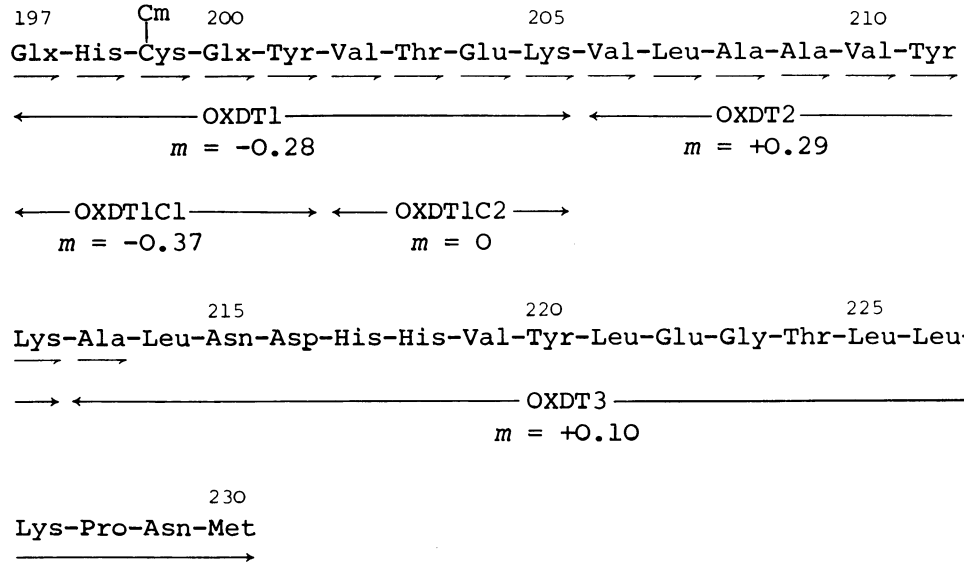

Fig. 3. *Derivation of the amino acid sequence of residues* 197–230 *of ox liver aldolase* [*numbered according to the rabbit muscle enzyme sequence given by Lai et al.* (1974)]

The tryptic peptides are aligned by homology with the rabbit muscle enzyme. The sequence of residues 213–230 is taken from Forcina & Perham (1971). → denotes a residue established by dansyl–Edman degradation. Residue 230 is shown as Met, but in the actual peptide studied this residue was present as homoserine.

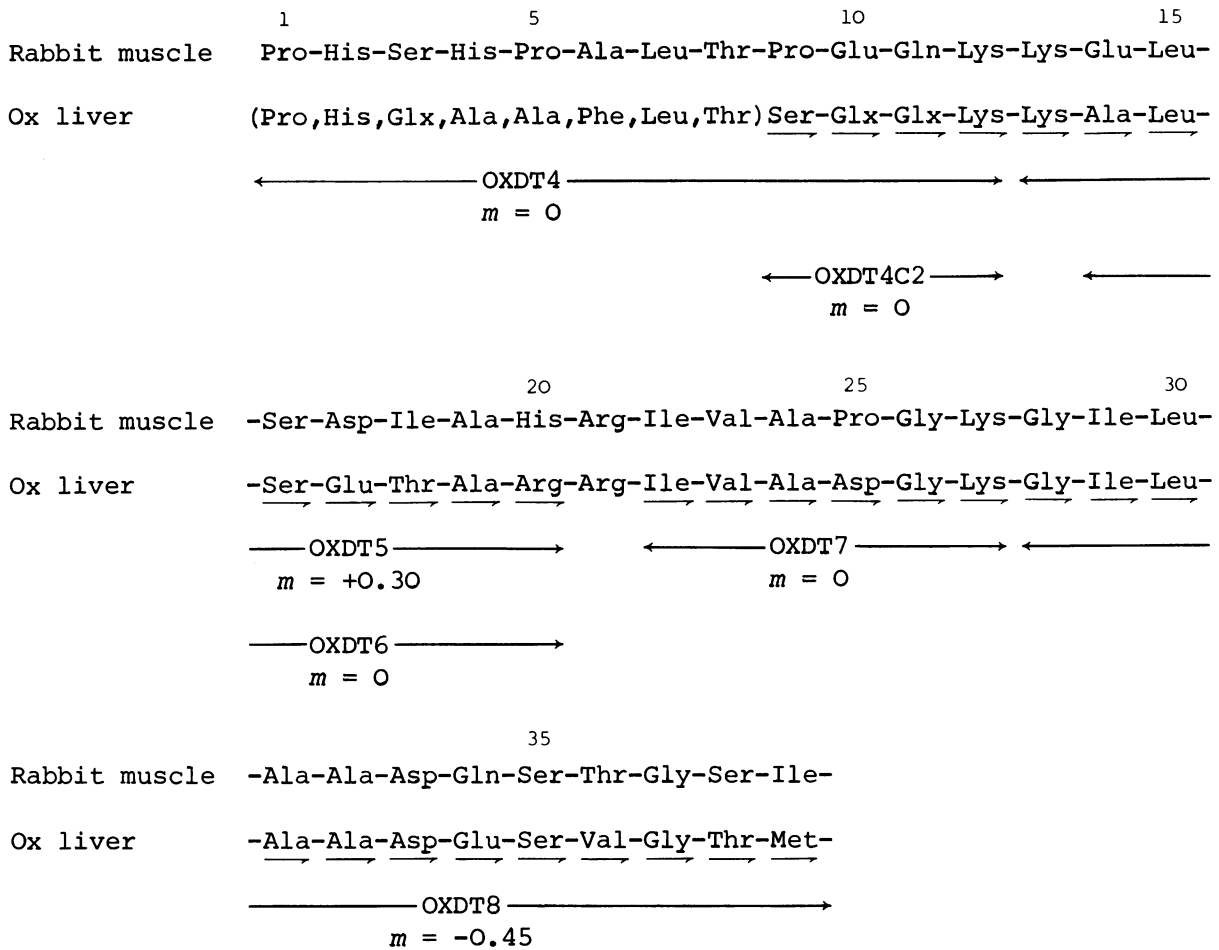

Fig. 4. *Derivation of the amino acid sequence of residues* 1–39 *of ox liver aldolase* [*numbered according to the rabbit muscle enzyme sequence given by Lai et al.* (1974)] The ox tryptic peptides are aligned by homology with the rabbit enzyme. → denotes a residue established by dansyl–Edman degradation. Residue 39 is shown as Met but in the actual peptide studied this residue was present as homoserine. The *N*-terminal residue of ox liver aldolase appears to be blocked.

with chymotrypsin, and the two product peptides (OXDT1C1 and OXDT1C2) were separated by paper electrophoresis at pH6.5. The amino acid sequences of these peptides were established by dansyl–Edman degradation. The sequence of amino acid residues 197–230 from ox liver aldolase is shown in Fig. 3, the peptides being aligned by homology with the rabbit muscle enzyme sequence. The amide assignments at positions 197 and 200 are left unresolved because the electrophoretic mobility of peptide OXDT1C1 ($m = -0.37$) did not allow an unequivocal answer.

Free lysine and arginine and further peptides were found in the tryptic digest of peak-D material. These peptides were also separated by paper electrophoresis at pH6.5 and 3.5. The amino acid compositions of these peptides (OXDT4, OXDT5, OXDT6, OXDT7 and OXDT8), from their homology with the rabbit muscle aldolase sequence, suggested that they might derive from the *N*-terminal region of the enzyme, and the amino acid sequences of peptides OXDT5, OXDT6, OXDT7 and OXDT8, established by means of dansyl–Edman degradation, confirmed this. Peptide OXDT4 showed no *N*-terminal residue when examined by the dansyl procedure. However, after chymotryptic digestion it yielded a peptide, OXDT4-C2, whose electrophoretic mobility ($m = 0$) and amino acid sequence Ser(Glu,Gln)Lys, determined by dansyl–Edman degradation, indicated beyond doubt that peptide OXDT4 is the *N*-terminal tryptic peptide from the enzyme. The *N*-terminal residue may well be blocked, since no free *N*-terminus could be detected for peptide OXDT4 and only glutamic acid was detected as *N*-terminal residue when the unfractionated peak D was examined, as reported above. The probable *N*-terminal amino acid sequence of ox liver aldolase, with some amide assignments left unmade and the *N*-terminal eight residues left in parentheses, is shown in Fig. 4, together with the *N*-terminal sequence of the rabbit muscle enzyme (Lai *et al.*, 1974), which was used to align the tryptic peptides and to place the free arginine found in the tryptic digest. The free lysine is accounted for by partial tryptic cleavage on either side of lysine-13.

The sequence analysis of the peptides in peak-D material rests on homology with the rabbit muscle enzyme. The results are wholly consistent with the presence of two peptides in peak D, comprising residues 1–39 and 179–230 of the ox liver enzyme. The elution position on the Sephadex G-75 column was compatible with these peptides each having about 35 amino acid residues, and the finding of only one *N*-terminal residue (glutamic acid) was reconciled by the subsequent sequence analysis of the tryptic peptides. The amino acid composition of peak-D material was consistent with it containing approximately equimolar amounts of two peptides comprising residues 1–39 and 197–230 of the ox liver enzyme shown in Figs. 3 and 4.

To extend the amino acid-sequence analysis of the ox liver aldolase on the *N*-terminal side of residue 197, tryptic peptides from decitraconylated material from peaks A, B and C were purified and analysed. A tryptic peptide, OXAT1, from peak-A material bore obvious similarity of amino acid composition to that of the region of residues 173–196 of the rabbit muscle

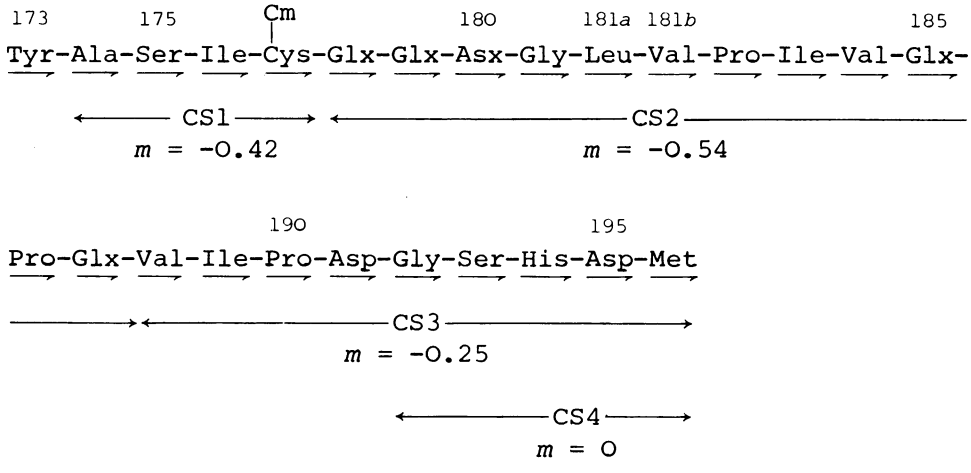

Fig. 5. *Derivation of the amino acid sequence of peptide* OXAT1 *(residues* 173–196*) of ox liver aldolase* [*numbered according to the rabbit muscle enzyme sequence given by Lai et al.* (1974)]

→ denotes a residue established by dansyl–Edman degradation. Residue 196 is shown as Met, but in the peptide studied this residue was present as homoserine.

enzyme (Lai *et al.*, 1974) and contained a homoserine residue at the *C*-terminus, proving that it must be the *C*-terminal tryptic peptide of a CNBr-cleavage product. The *N*-terminal sequence of the intact peptide was shown by means of dansyl–Edman degradation to be Tyr-Ala-Ser-Ile-CmCys-Glx-Glx-Asx-Gly-Leu-Val-Pro-Ile-Val-. The peptide was digested first with chymotrypsin and then with subtilisin, and four new peptides, together with free tyrosine, were purified by paper electrophoresis. The amino acid sequence of each of these peptides was completely determined by means of the dansyl–Edman degradation, which enabled an unequivocal sequence for peptide OXAT1 to be derived, with some amide assignments left unresolved. The results are shown in Fig. 5.

As expected, the amino acid-sequence homology with residues 173–196 of the rabbit muscle enzyme (Lai *et al.*, 1974) was strong, and the assignment of peptide OXAT1 to this region of the ox liver enzyme is beyond question. That residue 196 is methionine is fully consistent with the existence of another CNBr-cleavage fragment in peak-D material assigned to positions 197–230 (Fig. 3). However, to maintain the otherwise strong sequence homology with the rabbit muscle enzyme, it proved necessary to propose an insertion of a -Leu-Val- sequence at residues 181*a* and 181*b* (Fig. 5), in keeping with the comparable -Ile-Val- insertion proposed for the same position in the trout muscle enzyme (Fig. 2).

The amino acid sequence of residues 231–248 (adopting the present numbering system) of ox liver aldolase was determined by Forcina & Perham (1971). Taken together, the results from the present paper and from that earlier study enable us to propose an amino acid sequence (with certain amide assignments left unmade) for the region spanning residues 173–248, as shown in Fig. 8. Although the ordering of the peptides we have described rests on sequence homology with the rabbit muscle enzyme, the amino acid compositions of the peptides (parent and progeny) are readily reconciled with the formulated sequence and strongly imply that no peptides have been overlooked.

Amino acid-sequence studies on sturgeon muscle aldolase

The preparation of *S*-carboxy[^{14}C]methylated sturgeon muscle aldolase, its cleavage with CNBr and the separation of the citraconylated fragments by gel filtration on a column (114cm×2cm) of Sephadex G-75 in 0.5% NH_4HCO_3 were carried out as described above for trout muscle aldolase. The effluent was monitored for absorbance at 280nm and for radioactivity. The profile has been recorded previously (Anderson *et al.*, 1969; Gibbons *et al.*, 1970). The fragment designated SXB1 was purified further by an additional gel filtration on Sephadex G-50 in 0.5% NH_4HCO_3 (Gibbons *et al.*, 1970) and then decitraconylated. Full details of these operations are given in Supplementary Publication SUP 50098. We have already shown (Gibbons *et al.*, 1972) that on tryptic digestion this fragment SXB1 yields a peptide that comprises residues 213–230 of the sturgeon muscle enzyme [adopting once more the numbering of the rabbit muscle aldolase sequence proposed by Lai *et al.* (1974)].

The decitraconylated peak-SXB1 material was digested with trypsin, and the resulting peptides were separated by paper electrophoresis at pH6.5 and 3.5. Four peptides (SXB1T1, SXB1T2, SXB1T3 and SXB1T4) were recovered, together with free arginine. Peptide SXB1T4 was identified from its *N*-terminal residue (alanine) and its amino acid composition as the peptide comprising residues 213–230 of the sturgeon muscle enzyme whose sequence was reported by Gibbons *et al.* (1972). The amino acid sequences of peptides SXB1T2 and SXB1T3 were completely established by dansyl–Edman degradation. The sequence of the first 13 amino acid residues from the *N*-terminus of peptide SXB1T1 were established unequivocally by dansyl–Edman degradation, but that of the remaining seven residues was less certain. It did, however, fit the amino acid composition expected for these residues. The major problem appeared to be incomplete coupling or cleavage during the Edman degradation, leading to overlapping dansyl-amino acids at successive steps. A further difficulty was the existence of this peptide in several electrophoretic forms, all of which had exactly the same amino acid composition. The principal form had an electrophoretic mobility (*m*) of −0.32, compared with a minor form whose electrophoretic mobility was −0.60. This minor form, presumably a deamidation product of the main component, resolved into three further species on paper electrophoresis at pH3.5, presumably because of $\alpha \rightarrow \beta$ carboxyl shifts in two or more of the three -Asx-X- bonds in the molecule. These effects were not investigated further in view of the constancy of amino acid composition of the various forms of peptide SXB1T1, but they have prevented accurate assignment of amide groups.

The amino acid sequences of peptides SXB1T1, SXB1T2, SXB1T3 and SXB1T4, together with the free arginine found in the tryptic digest of fragment SXB1, correspond very well to residues 180–230 of the rabbit muscle enzyme sequence given by Lai *et al.* (1974). The presumed sequence of residues 180–230 of the sturgeon muscle enzyme is shown in Fig. 6, the peptides being aligned by homology with the rabbit muscle enzyme. As with the trout muscle and ox liver enzymes, it proved necessary to propose an insertion of two residues at position 181*a* and 181*b* to maximize the close sequence homology among these enzymes. The sum of the amino acid compositions of the tryptic peptides is in fair agreement with the amino acid composition of the starting

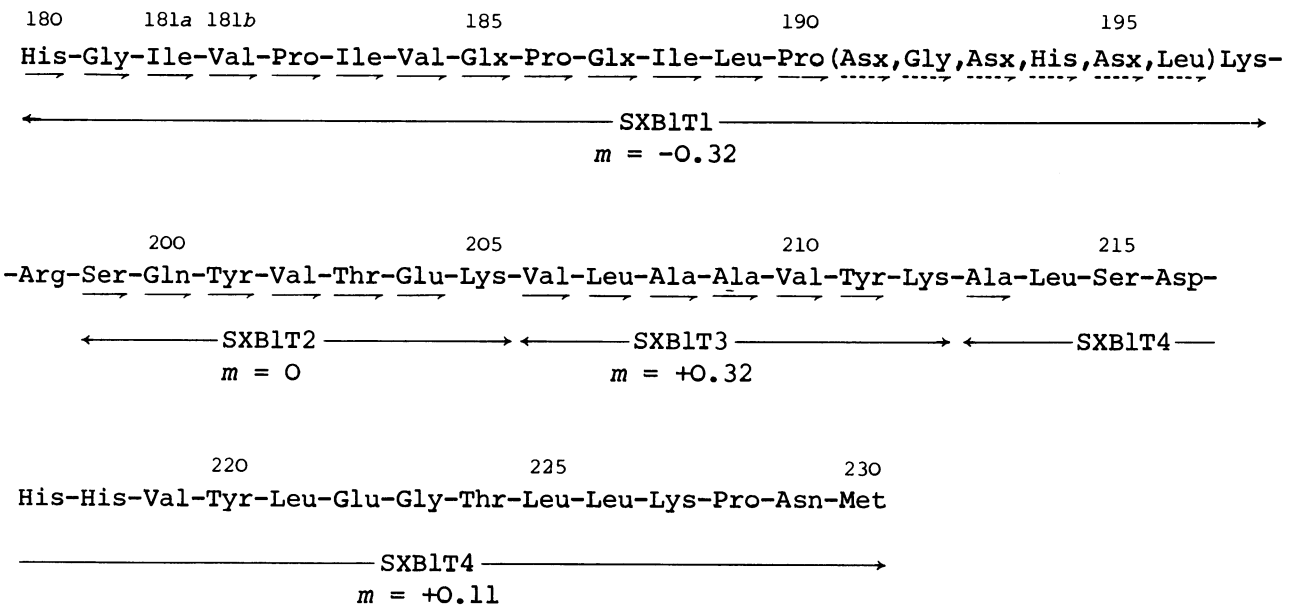

Fig. 6. *Derivation of the amino acid sequence of residues* 180–230 *of sturgeon muscle aldolase [numbered according to the rabbit muscle enzyme sequence given by Lai et al.* (1974)]

The ordering of peptides is based on the close sequence homology with the rabbit muscle enzyme. → denotes a residue established by dansyl–Edman degradation; --> denotes a residue whose placement by dansyl–Edman degradation was not unequivocal. The amino acid sequence of residues 213–230 is taken from Gibbons *et al.* (1972). Residue 230 is shown as Met, but in the actual peptide studied this residue was present as homoserine.

fragment SXB1. This and the strong sequence homology with the rabbit muscle enzyme strongly imply that no peptides have been overlooked.

The amino acid sequence of residues 231–248 for sturgeon (*Acipenser transmontanus*) muscle aldolase, adopting the present numbering system, has already been described (Gibbons *et al.*, 1972). Taken together, our results now enable us to propose an amino acid sequence for the region spanning residues 180–248 as shown in Fig. 8.

Amino acid-sequence studies on rabbit muscle aldolase

A recurrent finding in the work described above was the need to propose an insertion of two residues at positions 181*a* and 181*b* (-Ile-Val- for the trout and sturgeon muscle enzymes and -Leu-Val- for the ox liver enzyme) if homology with the rabbit muscle enzyme sequence proposed by Lai *et al.* (1974) were to be maximized. Similarly, in the rabbit muscle enzyme the sequence of residues 184 and 185 was reported to be -Glu-Val-, whereas in the other proteins we have studied the sequence turned out to be -Val-Glx- (Figs. 2, 5 and 6). A trytpic peptide spanning residues 173–198 of the rabbit muscle enzyme (Lai *et al.*, 1974) can easily be isolated from the relevant CNBr-cleavage peptide, RX3, described by Anderson & Perham (1970). We therefore isolated this peptide and determined its amino acid sequence from the *N*-terminus through to residue 185.

A sample (40mg) of rabbit muscle aldolase was *S*-carboxymethylated with iodo[2-^{14}C]acetic acid under denaturing conditions and then cleaved by treatment with CNBr. The resulting peptide fragments were citraconylated and gel-filtered on a column (114cm × 2cm) of Sephadex G-75 in 0.5% NH_4HCO_3. The effluent was monitored for absorbance at 280nm and for radioactivity. Peak-RX3 material (Anderson & Perham, 1970) was decitraconylated and digested with trypsin. The tryptic peptides were separated by paper electrophoresis at pH6.5, by paper chromatography and by paper electrophoresis at pH3.5. Peptide RX3T1 ($m = -0.24$) was readily detected by radioautography (Anderson & Perham, 1970). Its amino acid composition and *N*-terminal residue (tyrosine) confirmed its identity. The *N*-terminal sequence was established by dansyl–Edman degradation to be Tyr-Ala-Ser-Ile-CmCys-Glx-Glx-Asx-Gly-. Peptide RX3T1 was digested with subtilisin and three peptides (RX3T1S1, RX3T1S2 and RX3T1S3) were separated by paper electrophoresis at pH6.5 and 3.5. The amino acid sequences of peptides RX3T1S1 and RX3T1S3 were completely established and that of peptide RX3T1S2 was partly established by dansyl–Edman degradation. The electrophoretic mobilities of peptide RX3T1S3 allowed amide group assignments to be made for aspartic acid residues at positions 193 and 195, but this was not possible for peptide RX3T1S2 owing to its complexity. Peptides RX3T1S1 and RX3T1S2 were overlapped by the *N*-terminal sequence established for the intact peptide RX3T1. Peptide RX3T1S3 was placed by difference and also because it contained the *C*-terminal basic residue expected of a tryptic peptide.

The results are shown in Fig. 7. The amino acid sequence we propose is identical with that given by Lai *et al.* (1974) for the same region except for the insertion of the -Ile-Val- sequence at positions 181*a* and 181*b* and the inversion of -Glu-Val- to -Val-Glx- at positions 184 and 185 (see Fig. 8).

Supplementary information

Detailed evidence for the amino acid sequences described in this paper has been deposited

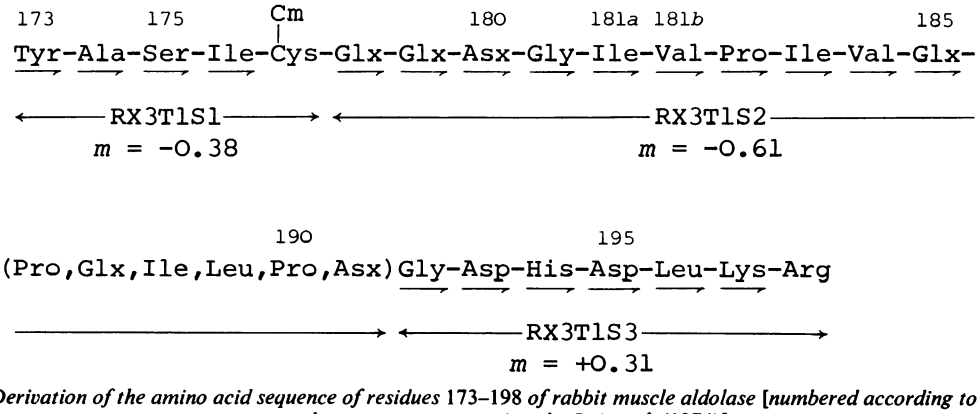

Fig. 7. *Derivation of the amino acid sequence of residues* 173–198 *of rabbit muscle aldolase* [*numbered according to the rabbit muscle enzyme sequence given by Lai et al.* (1974)]
—→ denotes a residue established by dansyl–Edman degradation.

as Supplementary Publication SUP 50098 at the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K. The information comprises the following.

(*a*) Details of the preparation of peptides TXF1, TXF2, TT3*a*, TT6*a* and TT6*b* from trout muscle aldolase, their amino acid compositions and secondary cleavages with other proteolytic enzymes, sequence analysis by dansyl–Edman degradation and assignment of amide groups from consideration of electrophoretic mobility at pH6.5.

(*b*) Details of the preparation of peptides OXDT1, OXDT2, OXDT3, OXDT4, OXDT5, OXDT6, OXDT7, OXDT8 and OXAT1 from ox liver aldolase, their amino acid compositions and secondary cleavages with other proteolytic enzymes, sequence analysis by dansyl–Edman degradation and assignment of amide groups from consideration of electrophoretic mobility at pH6.5.

(*c*) Details of the preparation of peptides SXB1T1, SXB1T2, SXB1T3 and SXB1T4 from sturgeon muscle aldolase, their amino acid compositions, sequence analysis by dansyl–Edman degradation and assignment of amide groups from consideration of electrophoretic mobility at pH6.5.

(*d*) Details of the preparation of peptide RX3T1 from rabbit muscle aldolase, its amino acid composition and secondary cleavage with subtilisin, sequence analysis by dansyl–Edman degradation and assignment of amide groups from consideration of electrophoretic mobility at pH6.5.

Discussion

The approach to comparative sequence analysis of aldolases that we adopted depends on close sequence homology between the proteins concerned. The early indications of this homology (Anderson *et al.*, 1969; Forcina & Perham, 1971) are fully borne out by the results summarized in Fig. 8. The amino acid sequence for rabbit muscle aldolase proposed by Lai *et al.* (1974) has been used as the comparator with which to align the peptide sequences determined for the other enzymes. No overlapping techniques have been employed in this work, but the closeness of the homology and the ability always to reconcile amino acid compositions with the results of dansyl–Edman degradation of individual peptides strongly suggest that no peptides have been overlooked. Residues 190–196 for the sturgeon muscle enzyme and 215–230 for the trout muscle enzyme are the only regions of sequence for which unequivocal dansyl–Edman degradations were not obtained.

The closeness of the homology led us in turn to reinvestigate the amino acid sequence of residues 181–185 of the rabbit muscle enzyme. Our results clearly indicate the need to insert the sequence -Ile-Val- between residues 181 and 182 and to reverse the order of residues 184 and 185. Both previously published sequences for the rabbit muscle enzyme (Lai *et al.*, 1974; Sajgo & Hajos, 1974) appear to be in error on these points. A likely reason for the omission of an -Ile-Val- sequence is the slow release of isoleucine and valine on acid hydrolysis, causing them to be missed from an amino acid composition. We found several regions of the sequence, e.g. peptides TT3*a* (Fig. 2), OXDT2 (Fig. 3), OXDT7 (Fig. 4), OXAT1 (Fig. 5), SXB1T1 and SXB1T3 (Fig. 6) and RX3T1 (Fig. 7), where the amino acid analysis after 24h hydrolysis indicated low values for isoleucine and valine, but the results of dansyl–Edman degradation were unequivocal. The sequence at positions 188–189 in all the aldolases examined except that of ox liver was -Ile-Leu-, but the -Val-Ile- sequence for the ox liver enzyme was strongly indicated by the dansyl–Edman degradation of peptide OXAT1CS3 (see Figs. 5 and 8). We consider it unlikely that the ox liver aldolase sequence is -Ile-Val- at this point.

In this region the two published amino acid sequences for rabbit muscle aldolase (Lai *et al.*, 1974; Sajgo & Hajos, 1974) differ in the states of amidation of glutamic acid residues at positions 179, 204 and 244 and of aspartic acid residues at positions 191 and 193. Reference to Fig. 8 suggests that Lai *et al.* (1974) are probably correct for aspartic acid-191 and aspartic acid-193, but that glutamic acid-204 and glutamic acid-244 are unamidated, as stated by Sajgo & Hajos (1974). On the other hand, our own amide assignments for aspartic acid-216, glutamic acid-222 and glutamic acid-245 differ from those agreed by Lai *et al.* (1974) and Sajgo & Hajos (1974). Amide assignments from electrophoretic mobility of peptides can be hard to make when a peptide contains histidine, and too much store should therefore not be placed on the results for such peptides. A more elaborate analysis will be necessary if any of these amide assignments turns out to have a crucial bearing on our understanding of the enzyme structure and mechanism.

The amino acid sequences summarized in Fig. 8 span 78 residues around the substrate-binding lysine residue of the various aldolases. Setting aside arguable differences and uncertainties of amide distribution, there are 12 differences of amino acid sequence between the muscle aldolases portrayed (positions 179, 180, 199, 210, 219, 233, 235, 236, 238, 239, 242 and 243). The differences are all of the more or less conservative type, e.g. isoleucine for valine, threonine for serine, asparagine for histidine. The clustering of seven of these 12 differences in the *C*-terminal 16 residues is a striking feature. If one extends the comparison to include the ox liver enzyme, the number of differences rises to 20 (positions 179, 180, 181*a*, 188, 189, 193, 196, 197, 198, 199, 210, 215, 219, 233, 236, 238, 239, 242, 243 and 246). The cluster of changes in

	173	175						180	181a	181b			185
Rabbit muscle[a]	Tyr-	Ala-	Ser-	Ile-	Cys-	Gln-	Gln-	Asn-	Gly-	—	—	-Pro-	Ile-	Glu-	Val-
Trout muscle	Tyr-	Ala-	Ser-	Ile-	Cys-	Gln-	Met-	His-	Gly-	Ile-	Val-	Pro-	Ile-	Val-	Glu-
Sturgeon muscle								His-	Gly-	Ile-	Val-	Pro-	Ile-	Val-	Glx-
Ox liver	Tyr-	Ala-	Ser-	Ile-	Cys-	Glx-	Glx-	Asx-	Gly-	Leu-	Val-	Pro-	Ile-	Val-	Glx-
Rabbit muscle[b]	Tyr-	Ala-	Ser-	Ile-	Cys-	Glx-	Glx-	Asx-	Gly-	Ile-	Val-	Pro-	Ile-	Val-	Glx-

	190							195						200
Rabbit muscle[a]	-Pro-	Glu-	Ile-	Leu-	Pro-	Asp-	Gly-	Asp-	His-	Asp-	Leu-	Lys-	Arg-	Cys-	Gln-
Trout muscle	-Pro-	Glu-	Ile-	Leu-	Pro-	Asx-	Gly-	Asx-	His-	Asx-	Leu-	Lys-	Arg-	Thr-	Glx-
Sturgeon muscle	-Pro-	Glx-	Ile-	Leu-	Pro	(Asx,Gly,Asx,His,Asx,Leu)						Lys-	Arg-	Ser-	Gln-
Ox liver	-Pro-	Glx-	Val-	Ile-	Pro-	Asp-	Gly-	Ser-	His-	Asp-	Met-	Glx-	His-	Cys-	Glx-
Rabbit muscle[b]	-(Pro,Glx,Ile,Leu,Pro,Asx)							Gly-	Asp-	His-	Asp-	Leu-	Lys-	Arg	

	205	210	215
Rabbit muscle[a]	-Tyr-Val-Thr-Gln-Lys-Val-Leu-Ala-Ala-Val-Tyr-Lys-Ala-Leu-Ser-		
Trout muscle	-Tyr-Val-Thr-Glx-Lys-Val-Leu-Ala-Ala-Met-Tyr-Lys-Ala-Leu(Ser,-		
Sturgeon muscle	-Tyr-Val-Thr-Glu-Lys-Val-Leu-Ala-Ala-Val-Tyr-Lys-Ala-Leu-Ser-		
Ox liver	-Tyr-Val-Thr-Glu-Lys-Val-Leu-Ala-Ala-Val-Tyr-Lys-Ala-Leu-Asn-		
Rabbit muscle[c]			-Ala-Leu-Ser-

	220	225	230
Rabbit muscle[a]	-Asn-His-His-Ile-Tyr-Leu-Gln-Gly-Thr-Leu-Leu-LYS-Pro-Asn-Met-		
Trout muscle	-Asx,His,His,Val,Tyr,Leu,Glx,Gly,Thr,Leu,Leu,LYS,Pro,Asx,Met)-		
Sturgeon muscle	-Asp-His-His-Val-Tyr-Leu-Glu-Gly-Thr-Leu-Leu-LYS-Pro-Asn-Met-		
Ox liver	-Asp-His-His-Val-Tyr-Leu-Glu-Gly-Thr-Leu-Leu-LYS-Pro-Asn-Met-		
Rabbit muscle[c]	-Asp-His-His-Ile-Tyr-Leu-Glu-Gly-Thr-Leu-Leu-LYS-Pro-Asn-Met-		

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                    235                 240                 245
Rabbit muscle[a]  -Val-Thr-Pro-Gly-His-Ala-Cys-Thr-Gln-Lys-Tyr-Ser-His-Gln-Gln-Ile-Ala-Met

Trout muscle      -Val-Thr-Ala-Gly-His-Ser-Cys-Ser-His-Lys-Tyr-Thr-His-Glx-Glx-Ile-Ala-Met

Sturgeon muscle   -Val-Thr-Ala-Gly-Gln-Ala-Cys-Thr-Lys-Lys-Tyr-Thr-Ala-Gln-Glu-Ile-Ala-Met

Ox liver          -Val-Thr-Ala-Gly-His-Ala-Cys-Thr-Lys-Lys-Tyr-Thr-Pro-Gln-Glu-Val-Ala-Met

Rabbit muscle[c]  -Val-Thr-Pro-Gly-His-Ala-Cys-Thr-Gln-Lys-Tyr-Ser-His-Glu-Glu-Ile-Ala-Met
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Fig. 8. *Proposed amino acid sequences for residues* 173–248 *for rabbit and trout muscle aldolases, for residues* 180–248 *for sturgeon muscle aldolase and for residues* 173–248 *for ox liver aldolase*

The rabbit muscle enzyme sequence marked *a* is taken from Lai *et al.* (1974), that marked *b* from the present paper (Fig. 7) and that marked *c* from Gibbons *et al.* (1970). Residues 190–196 in the sequence of the sturgeon muscle enzyme were not unequivocally established by dansyl-Edman degradation. The sequence of residues 215–230 for the trout muscle enzyme is assumed because of the identity of its composition with the corresponding sequence of sturgeon muscle aldolase. Lysine-227 is the residue that binds the substrate in imine linkage. For other details see the text.

the *C*-terminal region survives and is supplemented by a new cluster between positions 188 and 199. The number of differences between any two muscle enzymes (on average nine) is significantly lower than the number of differences between the ox liver and any muscle enzyme (on average 15), indicating an early evolutionary divergence of the genes for muscle and liver aldolases. Whether the cluster of changes between positions 188 and 199 has any significance for the well-known differences in specificity of muscle and liver aldolases must await a three-dimensional-structural analysis of these enzymes, since it is not discernible from the sequences alone.

On the basis of studies of the chemical reactivity of functional groups in aldolases, Lambert *et al.* (1977) concluded that the region of primary structure from residues 107–227 is likely to form part of a three-dimensional-structural feature, perhaps a folding domain, in these enzymes. Independently, Stellwagen (1976) proposed that residues 147–299 of rabbit muscle aldolase would comprise alternating segments of α-helix and β-strand resembling the 'super-secondary structure' of the NAD^+-binding domain common to several dehydrogenases. Our sequence work supports these ideas in two ways. First, the strong sequence homology shown in Fig. 8 is consistent with the preservation of structure, the two clusters of sequence changes having a majority of hydrophilic residues compatible with 'surface' loops. Secondly, in the *N*-terminal region, exemplified by the sturgeon muscle *N*-terminal sequence given by Anderson & Gibson (1973) and by Fig. 4, the rate of accepted mutations between aldolases from rabbit muscle, sturgeon muscle and ox liver is approximately twice that calculated for the interior region covered by Fig. 8. Further study of the *C*-terminal regions of ox liver and trout muscle aldolases (P. A. Benfield, B. G. Forcina & R. N. Perham, unpublished work) shows a pattern of mutation similar to that for the *N*-terminal region. Conservation of amino acid sequence in the middle tract of primary structures of the various aldolases is therefore high-lighted.

Sufficient information on the amino acid sequences of aldolases has now been collected for us to verify that the average rate of evolution of this enzyme is very similar to that of another glycolytic enzyme, glyceraldehyde 3-phosphate dehydrogenase, as forecast by Anderson *et al.* (1969). Defining a unit evolutionary period as the time in millions of years needed to establish a 1% difference between divergent lines, we calculate a unit evolutionary period for class-I aldolases to be about 20 million years, compared with a value of 18 million years for glyceraldehyde 3-phosphate dehydrogenase (Dickerson, 1971). Such a modest rate of mutation is likely to be a common feature of the evolution of glycolytic enzymes (Anderson *et al.*, 1969).

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