The complete amino acid sequence of human skeletal-muscle fructose-bisphosphate aldolase

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The complete amino acid sequence of human skeletal-muscle fructose-bisphosphate aldolase, comprising 363 residues, was determined. The sequence was deduced by automated sequencing of CNBr-cleavage, *o*-iodosobenzoic acid-cleavage, trypsin-digest and staphylococcal-proteinase-digest fragments. Comparison of the sequence with other class I aldolase sequences shows that the mammalian muscle isoenzyme is one of the most highly conserved enzymes known, with only about 2% of the residues changing per 100 million years. Non-mammalian aldolases appear to be evolving at the same rate as other glycolytic enzymes, with about 4% of the residues changing per 100 million years. Secondary-structure predictions are analysed in an accompanying paper [Sawyer, Fothergill-Gilmore & Freemont (1988) *Biochem. J.* 249, 789–793].

INTRODUCTION

Aldolases (EC 4.1.2.13) are a group of enzymes that catalyse reversible aldol cleavage/condensation reactions. The glycolytic aldolase is fructose-1,6-bisphosphate aldolase:

Aldolases can be classified into two groups that have strikingly different mechanisms of action. Class I aldolases form a Schiff-base intermediate between the C-2 carbonyl group of the substrate (dihydroxyacetone phosphate in the case of the glycolytic aldolase) and the ϵ -amino group of a lysine residue (reviewed by Horecker *et al.*, 1972). Class II enzymes, in contrast, do not form a covalent enzyme–substrate intermediate, and a bivalent transition-metal ion such as Zn^{2+} is required. Class II aldolases occur primarily in bacteria and yeast (Rutter, 1964; Warburg & Christian, 1943).

Fructose-bisphosphate aldolase isolated from vertebrates, insects and higher plants is a tetrameric enzyme with essentially identical subunits of M_r 40000. In mammalian tissues aldolase exists in three main forms: A predominates in skeletal muscle, B in liver and kidney, and C, together with a variety of hybrids with A and B subunits, in brain. Complete amino acid sequences are available for fructose-bisphosphate aldolase from rabbit muscle (Tolan *et al.*, 1984), rat liver (Tsutsumi *et al.*, 1983), *Drosophila melanogaster* (Malek *et al.*, 1985) and

Trypanosoma brucei (Clayton, 1985). Partial (but extensive) sequences of the human liver enzyme have also been published (Besmond *et al.*, 1983; Costanzo *et al.*, 1983). Crystallographic studies on aldolase from human muscle, rabbit muscle and *D. melanogaster* have been undertaken, and low-resolution structures have been published (Millar *et al.*, 1981; Sygusch *et al.*, 1985;

Brenner-Holzach & Smit, 1982). Work is in progress by all these groups to extend the crystallographic studies to high resolution.

We now report here the determination of the complete amino acid sequence of human skeletal-muscle aldolase. The availability of this sequence allows the rate of evolution of the muscle isoenzyme to be compared with that of the liver isoenzyme, and shows that the muscle isoenzyme is much more strongly conserved. An analysis of the predicted secondary structures of the available aldolase primary structures is presented in the accompanying paper (Sawyer *et al.*, 1988). The sequence information will be of importance for the interpretation of the crystallographic structure.

EXPERIMENTAL

Purification and chemical modification of human skeletal-muscle aldolase

The purification and carboxymethylation procedures are described in detail in Freemont *et al.* (1984).

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Succinylation (2-carboxypropionylation) was done by the addition of a 200-fold molar excess of succinic anhydride (general-purpose-reagent quality from BDH Chemicals) over lysine residues as described by Russell *et al.* (1986).

Purification of fragments for sequencing

The methods for CNBr and *o*-iodosobenzoic acid cleavage, and the results of gel filtration of the fragments, are given in Freemont *et al.* (1984). The details of the conditions used for digestion of fragment CN2 with staphylococcal proteinase or with trypsin, and for digestion of aldolase with trypsin, are given in Figs. 1, 2, 3 and 4 respectively. Peptides were purified by h.p.l.c. on Waters Associates C₁₈ μ Bondapak columns (0.4 cm $\times$ 30 cm), with Waters pumps, gradient controller and detector. The flow rate was 1 ml/min, and peaks were collected manually. Some peptides were further purified on a Waters phenyl μ Bondapak column (0.4 cm $\times$ 30 cm). The separations were done with 0.1% (v/v) trifluoroacetic acid as solution A, and acetonitrile/propan-2-ol/methanol (1:1:1, by vol.) as solution B. Enzymes were from Worthington Biochemical Corp., and solvents were from Rathburn Chemicals (Walkerburn, Peeblesshire, Scotland, U.K.).

Amino acid analysis and sequence determination

The procedures for amino acid analysis are given in Freemont *et al.* (1984). Sequencing was done automatically with a Beckman 890C liquid-phase sequencer equipped with the Beckman cold-trap accessory as described in Russell *et al.* (1986).

RESULTS

Sequencing strategy

Fragments of aldolase were generated by cleavage with CNBr, *o*-iodosobenzoic acid and proteolytic enzymes as outlined in Scheme 1. The results of the

purification of the CNBr- and *o*-iodosobenzoic acid-cleavage fragments and the *N*-terminal sequence analyses of these fragments are presented in Freemont *et al.* (1984). In the present paper we report the purification and sequencing of the proteolytic fragments necessary to provide overlaps between the chemically derived fragments and to complete the sequence determination.

Peptides derived from fragment CN2

Carboxymethylated fragment CN2 (600 nmol) was digested with staphylococcal proteinase under conditions specific for cleaving peptide bonds C-terminal to glutamic acid residues (Drapeau *et al.*, 1972) as described in Fig. 1, and the resulting peptides were separated on a C₁₈ μ Bondapak reverse-phase column (Fig. 1). Eighteen peptides were successfully isolated by this method, and a further two peptides were purified after an additional separation of the peak 13 material on a phenyl μ Bondapak column (Fig. 1 inset). Eight of these peptides (SP19, SP3, SP12, SP9, SP8, SP11, SP1 and SP16) were completely sequenced as shown in Fig. 5. The initial yields were generally about 15 nmol (range 5–30 nmol), and the repetitive yields were between 85 and 90%, with one exception. Peptide SP8 had a low repetitive yield (69%) because of extensive loss of material from the spinning cup after cycle 8. There was good agreement between the amino acid compositions of the peptides and the corresponding amino acid sequences (Freemont, 1984).

There was extensive cleavage adjacent to aspartate residues despite the use of conditions favouring specific cleavage at glutamate residues. Thus peptides SP13F4, SP3, SP9, SP1 and SP16 all resulted from cleavage at aspartate residues (Fig. 5). In addition, an unusual cleavage of a Ser–Lys peptide bond at residues 99–100 was observed. A similar type of cleavage has been reported for horse pancreas phospholipase A₂ (Evenberg *et al.*, 1977).

Carboxymethylated and succinylated fragment CN2

Scheme 1. Fragmentation of aldolase

The cleavage and digestion procedures and the methods of purification are described in the text. Abbreviations: IOBA, *o*-iodosobenzoic acid; R.p. h.p.l.c., reverse-phase h.p.l.c.; S. proteinase, staphylococcal proteinase.

Fig. 1. Separation of staphylococcal-proteinase-digest peptides from fragment CN2 by h.p.l.c.

Freeze-dried fragment CN2 (600 nmol) was dissolved in 1 ml of 50 mM- NH_4HCO_3 , pH 7.8, digested with 0.2 mg of staphylococcal proteinase for 18 h at 37 °C, freeze-dried and redissolved in 0.8 ml of 0.1 % (v/v) trifluoroacetic acid. The peptides in a 0.15 ml sample were separated on a C_{18} μ Bondapak column as described in the text. The inset shows the separation of peptides in peak 13 on a phenyl μ Bondapak column. Fractions corresponding to each major peak were collected. —, A_{214} ; -----, concn. of solution B.

Fig. 2. Separation of trypsin-digest peptides from succinylated fragment CN2 by h.p.l.c.

Freeze-dried succinylated fragment CN2 (250 nmol) was dissolved in 0.5 ml of 1 % (w/v) NH_4HCO_3 , pH 7.8, digested with 0.25 mg of trypsin for 4 h at 37 °C, freeze-dried and redissolved in 0.4 ml of 0.1 % (v/v) trifluoroacetic acid. The peptides in a 0.1 ml sample were separated on a C_{18} μ Bondapak column as described in the text. Fractions corresponding to each major peak were collected. —, A_{214} ; -----, concn. of solution B.

(250 nmol) was digested with trypsin, and the peptides were separated on a C_{18} μ Bondapak column (Fig. 2). Thirteen peptides were obtained, and three of these (ST13, ST8 and ST11) were subjected to *N*-terminal sequence analysis (Fig. 5). Peptide ST13 was a long peptide of 65 residues, and the *N*-terminal 52 residues were successfully identified (initial yield 13 nmol, repetitive yield 94%). A comparison of the amino acid composition of this peptide with the sequence shows a substantial discrepancy for valine: there are eight valine residues in the sequence, and only 4.8 in the composition. An inspection of the sequence shows that there are two Val-Val and two Val-Ile sequences. The low values in the composition were probably a result of incomplete hydrolysis of the peptide bonds between these residues. Peptides ST8 and ST11 had initial yields of about 10 nmol, and repetitive yields of 90% and 80% respectively. There was good agreement between the amino acid compositions of the tryptic peptides (other than ST13) and the corresponding amino acid sequences (Freemont, 1984).

Peptides derived from fragment CN4

Carboxymethylated fragment CN4 was digested with trypsin, and the peptides were separated by reverse-phase h.p.l.c. (Fig. 3). Seven peptides were purified, and the sequence of peptide T7 was determined to complete the sequence of fragment CN4 (Fig. 5). The initial yield was 20 nmol, and the repetitive yield 83%. The amino acid compositions of the other peptides agreed well with the corresponding sequences (Freemont, 1984), except that peptide T4 had low values of valine and isoleucine (1.2 residues of valine instead of 2, and 2.8 residues of isoleucine instead of 4). Inspection of the sequence shows the peptide has two Ile-Val sequences and one Ile-Leu. It

is likely that this is another example of incomplete hydrolysis of resistant peptide bonds.

Trypsin digestion of aldolase

Trypsin-digest peptides were isolated from aldolase primarily to obtain methionine-containing peptides to establish overlaps between the CNBr-cleavage fragments. The conditions of digestion and the separation of the peptides by reverse-phase h.p.l.c. are given in Fig. 4. Three of the 40 peptides (AT36, AT31 and AT32) contained methionine, and were thus sequenced (Fig. 5). These results provided unequivocal evidence for the order of the CNBr-cleavage fragments. Peptide AT27 was also sequenced to provide overlap information between peptides SP8 and SP11. The initial yields were between 15 and 50 nmol, and the repetitive yields between 88 and 92%.

Generally there was good agreement between the amino acid compositions of the peptides and their sequences (Freemont, 1984). There were three exceptions. The most significant of these concerns peptide AT28, which had a low value of tyrosine: 1.5 residues instead of 2. This peptide corresponds to the *C*-terminal 22 residues of aldolase. It has been known for some time that the *C*-terminal tyrosine residue of aldolase is essential for activity (Drechsler *et al.*, 1959), and that the enzyme can be inactivated by carboxypeptidase digestion (Rutter *et al.*, 1961). The low value of tyrosine in peptide AT28 would be consistent with the fact that the aldolase used in these studies had a lower specific activity than expected (about 5 units/mg instead of about 15 units/mg) (Freemont *et al.*, 1984). It is likely that the final tyrosine residue was missing from a proportion of our sample of aldolase, probably because of limited carboxypeptidase digestion in the muscle before fractionation or during the early purification steps. Further justification for this

Fig. 3. Separation of trypsin-digest peptides from fragment CN4 by h.p.l.c.

Freeze-dried fragment CN4 (400 nmol) was dissolved in 0.3 ml of 1% (w/v) NH_4HCO_3 , pH 7.8, digested with 0.03 mg of trypsin for 4 h at 37 °C, freeze-dried and redissolved in 0.4 ml of 1% (w/v) NH_4HCO_3 , pH 7.5. The peptides in a 0.05 ml sample were separated on a C_{18} μ Bondapak column as described in the text, except that solution A was 1% NH_4HCO_3 . Fractions corresponding to each major peak were collected. —, A_{214} ; ----, concn. of solution B.

Fig. 4. Separation of trypsin-digest peptides from aldolase by h.p.l.c.

Freeze-dried carboxymethylated aldolase (200 nmol of subunit) was dissolved in 0.8 ml of 1% (w/v) NH_4HCO_3 , pH 7.8, digested with 0.08 mg of tosylphenylalanylchloromethane ('TPCK')-treated trypsin for 2 h at 37 °C, freeze-dried and redissolved in 0.4 ml of 0.1% (v/v) trifluoroacetic acid. The peptides in a 0.1 ml sample were separated on a C_{18} μ Bondapak column as described in the text. Fractions corresponding to each major peak were collected. —, A_{214} ; ----, concn. of solution B.

suggestion comes from peptide AT22, which is six residues shorter than peptide AT28, but otherwise identical. This peptide was isolated in relatively low yield, and probably is derived from carboxypeptidase-degraded aldolase.

The other two peptides with discrepancies between their compositions and sequences were AT31 and AT40. The former peptide had a low leucine content, which is consistent with the presence of a Leu-Leu sequence. The latter peptide had low values of serine and threonine. These residues were identified unambiguously by sequencing, and it is not clear why the composition values were low.

Complete sequence of human skeletal-muscle aldolase

The complete amino acid sequence of the enzyme, comprising 363 residues, is presented in Fig. 5. The subunit M_r value calculated from the sequence is 39262; this value is close to that estimated by physical methods. The order of the CNBr- and *o*-iodosobenzoic acid-cleavage fragments was established by the purification and sequence determination of staphylococcal-proteinase- and trypsin-digest peptides. Most of the sequence of the enzyme was established from two or more independently isolated and sequenced fragments. In a few cases only a single sequence was determined, which was substantiated by the compositions of additional peptides derived from the same region. In all these cases the phenylthiohydantoin derivatives of the amino acid residues could be identified unambiguously.

DISCUSSION

The complete sequence of human skeletal-muscle fructose-bisphosphate aldolase is summarized in Fig. 6, and is compared with the sequences of aldolase from rabbit skeletal muscle, *Trypanosoma brucei*, *Drosophila melanogaster*, rat liver and human liver. It is apparent

that the sequences are highly conserved, especially when the evolutionary distances between mammals, protozoa and insects are taken into consideration. The overall diversity among the sequences is given in Table 1. The observed sequence differences are corrected to PAMs (accepted point mutations) to take account of superimposed and back mutations. One of the most striking features is that the rate of evolution is very low. This is particularly true of the mammalian muscle (or A) isoenzyme, which is tolerating only about 2 PAMs/100 million years. It is thus changing at a much lower rate than, for example, ribonuclease, which has a rate of mutation acceptance about 20 times greater, or trypsin, which is changing about 6 times faster. By contrast, the liver (or B) isoenzyme of aldolase is changing about 5 times faster than the muscle isoenzyme. The overall rate of evolution of aldolase is about 4–6 PAMs/100 million years, and is typical of most of the glycolytic enzymes. This rate is the same as the small, highly specialized, cytochrome *c* molecule.

It is possible that the muscle isoenzyme is so highly conserved because it experiences many constraints in addition to the need to maintain catalytic function. It is, for example, present in high concentration in a tissue that is itself evolving only very slowly. Muscle aldolase is known to interact with the highly conserved F-actin molecule (Arnold & Pette, 1968) and with phosphofructokinase (Hofer *et al.*, 1987). It is important to note in this context that the different isoenzyme sequences from a single species can be strikingly different. Thus the human muscle and human liver aldolase sequences are as divergent from each other as they are from *D. melanogaster* aldolase. This would imply that the gene duplication event giving rise to the different isoenzymes either took place early in vertebrate evolution, or that a more recent gene duplication event was followed by a period of exceptionally rapid change.

Comparison of the sequence of *D. melanogaster*

Fig. 5. Amino acid sequence of human skeletal-muscle aldolase

The continuous lines (—) indicate those residues identified by automated *N*-terminal sequence analysis, and the dotted lines (·····) the unsequenced regions. Peptide nomenclature is given in Scheme 1, and details of the methods are given in the text.

aldolase with those of the mammalian isoenzymes shows that it is more similar to the muscle isoenzyme. This would indicate that the ancestral form corresponded more closely with the muscle isoenzyme, and that the liver isoenzyme arose by gene duplication. It seems likely that the rate of aldolase evolution in non-mammalian tissues is 4–6 PAMs/100 million years, and that after the gene duplication event the muscle isoenzyme has evolved more slowly and the liver isoenzyme more rapidly. (This type of information is not apparent from the *T. brucei* sequences, as the protozoan is too evolutionarily distant, and the isoenzyme differences are swamped by the large number of total differences.)

The comparisons that have been considered in Table 1

have involved the average differences between entire amino acid sequences, and are appropriate for estimating evolutionary divergence. Of course, sequence variations are not randomly distributed, and certain regions can be exceptionally conserved or exceptionally variable.

A number of residues have been implicated from chemical modification studies on Class I fructose-bisphosphate aldolases as being important for activity, and with one exception these residues are conserved in all the sequences. Lys-229 forms a Schiff-base intermediate with the substrate dihydroxyacetone phosphate (Horecker *et al.*, 1961), and is thus clearly essential for activity. It is present in a strongly conserved region. Arg-55 and Lys-146 are both implicated as binding the C-1

Fig. 6. Comparison of the amino acid sequences of human, rabbit, rat, *Drosophila melanogaster* and *Trypanosoma brucei* aldolases

The sequences are available in the following publications: Rab A, rabbit muscle (Tolan *et al.*, 1984); T. b., *T. brucei* (Clayton, 1985); D. m., *D. melanogaster* (Malek *et al.*, 1985); Rat B, rat liver (Tsutsumi *et al.*, 1983); Hum B, human liver (partial sequences) (Costanzo *et al.*, 1983; Besmond *et al.*, 1983). The residues are numbered in accordance with the human muscle sequence. The symbol – indicates that a gap has been introduced to maximize homology, and the symbol ★ shows the residues implicated as being important for activity. Residues that are identical in three or more sequences are boxed in.

Table 1. Evolution of aldolase

The number of residues compared relates only to known matched sequences; deletions and compositions of unsequenced peptides are not included. The sequences are available in the references given in the legend to Fig. 6. Values for accepted point mutations (PAM) and estimates of times since divergence are from Dayhoff (1978). Entries in parentheses are particularly speculative estimates, but nevertheless give an indication that they are consistent with an overall rate of evolution of 4–6 PAMs/100 million years.

Sequences compared	No. of residues compared	Differences		PAM	Time since divergence (10 ⁶ years)	PAM/100 × 10 ⁶ years
		(no.)	(%)			
Hum A–Rab A	363	5	1.4	1.4	75	1.9
Hum B–Rat B	164	13	7.9	8.0	75	11
D. m.–Hum A	360	107	29	37	900	4.3
D. m.–Hum B	161	58	36	50	900	5.6
D. m.–Rat B	360	129	36	50	900	5.6
T. b.–Hum A	360	185	51	86	(1500)	(5.7)
T. b.–Hum B	162	83	51	86	(1500)	(5.7)
T. b.–Rat B	360	192	53	92	(1500)	(6.1)
T. b.–D. m.	357	189	53	92	(1500)	(6.1)
Hum A–Hum B	164	59	35	48	–	–
Hum A–Hum B	363	117	32	43	–	–

phospho group of both fructose biphosphate and dihydroxyacetone phosphate (Lubini & Christen, 1979; Pathy *et al.*, 1979), and both amino acid residues are conserved in all species. As already discussed, Tyr-363 is essential for activity, and it too is invariant in all species. Photo-oxidation studies have indicated that histidine is important in the catalytic mechanism (Hoffee *et al.*, 1967; Davis *et al.*, 1970), and chemical modification with *N*-bromoacetyl ethanolamine phosphate has enabled a

labelled peptide containing alkylated His-361 to be isolated (Hartman & Welch, 1974). Horecker *et al.* (1972) have proposed that His-361 catalyses the removal and addition of a proton from and to C-3 of the enzyme-bound dihydroxyacetone phosphate complex. However, this residue is not conserved in the human and rat liver isoenzyme sequences, nor in the *T. brucei* enzyme, and it is therefore unlikely that it plays a crucial role in the catalytic mechanism.

The *N*-terminal region of aldolase is exceptionally variable, and, for example, three of the five differences between human and rabbit muscle aldolase involve residues 2–4. The *T. brucei* sequence has a ten-residue *N*-terminal extension, which may be important for ensuring that the enzyme is correctly addressed for localization within the glycosome organelle found in *Trypanosoma* (Clayton, 1985). Why should the terminal regions be so variable? A possible explanation may relate to the fact that terminal regions are usually not buried in the protein structure. Thus these portions of an enzyme may vary both in amino acid sequence and in length without disrupting the conserved tertiary structure that is required for activity. In the case of the *T. brucei* enzyme, variation of the terminal region can be correlated with the 'acquisition' of additional properties.

It is relevant to note that chicken muscle aldolase isolated in the presence of proteolytic inhibitors had an additional residue, *a*-*N*-blocked methionine, at the *N*-terminus (Lebherz *et al.*, 1984). Aldolase from *Drosophila* also has been shown to have a blocked *N*-terminus, in this case an *a*-*N*-acetylated threonine (Malek *et al.*, 1985). It would seem likely that samples of aldolase purified from muscle by traditional methods suffer the proteolytic removal of the blocked *N*-terminal residue, which may account for the unblocked proline *N*-terminus observed in these studies. The presence or absence of a blocked *N*-terminus appears to have little functional significance, as the specific catalytic activities and thermostabilities of the two forms of chicken muscle aldolase are similar (Lebherz *et al.*, 1984).

A number of other portions of aldolase are exceptionally variable: for example, residues 39–48, 63–72, 91–99, 108–120, 154–164 and 343–358. All of these regions appear to correspond to portions of the polypeptide chain lying between elements of predicted regular secondary structure, and may represent surface loops. Residues 91–99 are of particular interest because they are strongly predicted as α -helix in the *D. melanogaster* and rat liver sequences but not in the others. The results of secondary-structure predictions on class I fructose-bisphosphate aldolases are presented and discussed in the accompanying paper (Sawyer *et al.*, 1988).

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