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The complete amino acid sequence of chicken skeletal-muscle enolase

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The complete amino acid sequence of chicken skeletal-muscle enolase, comprising 433 residues, was determined. The sequence was deduced by automated sequencing of hydroxylamine-cleavage, CNBr-cleavage, *o*-iodosobenzoic acid-cleavage, clostripain-digest and staphylococcal-proteinase-digest fragments. The presence of several acid-labile peptide bonds and the tenacious aggregation of most CNBr-cleavage fragments meant that a commonly used sequencing strategy involving initial CNBr cleavage was unproductive. Cleavage at the single Asn–Gly peptide bond with hydroxylamine proved to be particularly useful. Comparison of the sequence of chicken enolase with the two yeast enolase isoenzyme sequences shows that the enzyme is strongly conserved, with 60% of the residues identical. The histidine and arginine residues implicated as being important for the activity of yeast enolase are conserved in the chicken enzyme. Secondary-structure predictions are analysed in an accompanying paper [Sawyer, Fothergill-Gilmore & Russell (1986) Biochem. J. **236**, 127–130].

INTRODUCTION

Enolase (EC 4.2.1.11) catalyses the only dehydration reaction in glycolysis:

$$CH_2OH\text{–}CHOPO_3^{2-}\text{–}CO_2^- \rightleftharpoons CH_2{=}COPO_3^{2-}\text{–}CO_2^- + H_2O$$

The enzyme is active as a dimer with identical subunits of M_r 45000, and requires Mg^{2+} for the stability of the dimer (reviewed by Wold, 1971). Additional Mg^{2+} ions are required for catalysis, and the 'active enzyme can thus be considered to be a metal-activated metalloprotein' (Chin *et al.*, 1981*a*). The activity of enolase has been extensively studied, and, for example, spectroscopic measurements in the presence or in the absence of Mg^{2+}, substrate and/or inhibitors have provided many clues about the stereochemistry of the ligand-binding sites (reviewed by Brewer, 1981). Chemical modification studies have implicated histidine (Westhead, 1965; Elliot & Brewer, 1979), carboxy (Rose & O'Connell, 1969; Russell & Fothergill, 1982) and arginine (Riordan *et al.*, 1977; Elliott & Brewer, 1978) residues as being important for activity.

Three tissue-specific isoenzymes of enolase exist in vertebrate tissues (Rider & Taylor, 1975*a*,*b*), and much interest has been stimulated by the discovery that the presence of brain-specific enolase in the serum could provide a useful marker for tumours of neuronal tissue (Carney *et al.*, 1982). Yeast (*Saccharomyces cerevisiae*) also contains enolase isoenzymes (Malmström, 1957), and the two genes coding for enolase have been isolated and sequenced (Holland *et al.*, 1981). Most of the amino acid sequence of the readily purified A isoenzyme of yeast has also been determined (Chin *et al.*, 1981*a*,*b*).

High-resolution X-ray-crystallographic structures are available for almost all glycolytic enzymes (reviewed by Fothergill-Gilmore, 1986). Enolase is a notable exception, primarily because attempts to obtain suitable crystals of yeast enolase have thus far been unsuccessful (Brewer, 1981). However, crystals of chicken skeletal-muscle enolase have recently been shown to be suitable for high-resolution X-ray-diffraction studies (H. C. Watson & P. J. Shaw, personal communication), and work is commencing to solve the structure. We report here the determination of the complete amino acid sequence of chicken skeletal-muscle enolase. A comparison with the yeast enolase sequence shows these enzymes to be strongly conserved. An analysis of the predicted secondary structure of enolase is presented in an accompanying paper (Sawyer *et al.*, 1986).

EXPERIMENTAL

Purification of chicken enolase

The purification procedure was based on that of Scopes (1977*a*,*b*), and the details of the affinity-elution step are given in Fig. 1. Enolase activity was assayed by measuring the formation of phosphoenolpyruvate by the increase in absorbance at 230 nm (Westhead, 1966).

Purification of CNBr-cleavage, *o*-iodosobenzoic acid-cleavage and hydroxylamine-cleavage fragments

The details of the cleavage procedures and the results of gel filtration of the fragments are given in Figs. 2, 3 and 5.

Carboxymethylation and succinylation

Carboxymethylation was done under reducing conditions essentially as described by Crestfield *et al.* (1963). The enolase (up to 5 μmol of subunit) was dissolved in 8 M-urea/0.5 M-Tris/HCl/2 mM-EDTA buffer, pH 8.5. A 2.5-fold molar excess of dithiothreitol over free thiol groups was added, and the solution was left under N_2 for 4 h at room temperature. Reaction with a 5.5-fold molar excess of iodoacetic acid was for 15 min in the dark at

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room temperature, and was stopped by the addition of 1 drop of 2-mercaptoethanol. The carboxymethylated enolase was dialysed against 1% (w/v) NH_4HCO_3 and then freeze-dried.

Succinylation (2-carboxypropionylation) was done by the addition of a 200-fold molar excess of succinic anhydride over lysine residues. Dry succinic anhydride was added to the sample of enolase or peptide (about 5 mg/ml) at 30 min intervals over 5 h. The solution was stirred, and the pH was kept above 9 with Tris buffer. (Tris, being a rich source of amino groups, may not be the best choice of buffering agent, but in our hands the succinylation of samples proceeded satisfactorily to completion despite its presence.) The succinylated sample was dialysed against 1% (w/v) NH_4HCO_3 and then freeze-dried.

H.p.l.c. purification of clostripain-digest and staphylococcal-proteinase-digest peptides

The details of the conditions used for digestion with clostripain and with staphylococcal proteinase are given in Figs. 4 and 6 respectively. Peptides were purified by h.p.l.c. on Waters Associates C_{18} μBondapak columns, with Waters pumps, gradient controller and detector. Peaks were collected manually. Some peptides were further purified on a Waters phenyl μBondapak column. Most of the separations were done with 0.1% trifluoroacetic acid as solution A and acetonitrile/propan-2-ol/methanol (1:1:1, by vol.) as solution B. Occasionally propan-2-ol was used as solution B, although the high back pressure at high concentrations of propan-2-ol was a problem. In the early stages of this work, an elution system of 0.1% NH_4HCO_3 as solution A and acetonitrile as solution B gave good separations. It was then realized that the useful life of the h.p.l.c. columns was unacceptably short, presumably because the pH of the NH_4HCO_3 solution was sufficiently high to damage the column packing material. The NH_4HCO_2 solution, can be adjusted to below pH 8 to give longer column life, but the pH rises rapidly (within an hour) to unacceptably high values.

Amino acid analysis

Hydrolysis was done in evacuated tubes with 0.5 ml of 5.7 M-HCl (AristaR HCl diluted with an equal volume of water) at 110 °C for 20, 48 or 96 h. Quantitative analysis was carried out with a Waters amino acid analysis h.p.l.c. system incorporating a Waters ion-exchange column and fluorescence detection of amino acids after reaction with hypochlorite (to convert proline into a primary amine) and *o*-phthalaldehyde.

Amino acid sequence determination

Sequencing was done automatically with a Beckman 890C liquid-phase sequencer or with an Applied Biosystems gas-phase sequencer. The liquid-phase sequencer was equipped with the Beckman cold-trap accessory, and was operated with a double-cleavage double-extraction programme developed by Dr. Jean Gagnon from the method of Hunkapiller & Hood (1978). Polybrene (3 mg) was added to the cup before each sample (Klapper *et. al.* 1978). The thiazolinones were extracted from the spinning cup with butyl chloride containing dithioerythritol (10 mg/l) and tributylphosphine (100 μl/l) (Frank, 1979). Samples were collected into tubes containing 0.2 ml of freshly prepared 0.1 M-HCl containing 0.1% (v/v) ethanethiol. Solvent was blown off by a stream of N_2 at room temperature, and the aqueous layer that remained was then heated at 80 °C for 10 min, frozen and dried under vacuum at room temperature. The phenylthiohydantoin derivatives were identified by h.p.l.c. on Waters C_{18} Resolve columns (0.39 cm × 15 cm) with an acetate buffer (pH 5.0)/acetonitrile gradient system and a 254 nm detector (Russell, 1984). Repetitive yields were calculated by linear-regression analysis of the yields at each cycle (omitting the serine, threonine, isoleucine and *S*-carboxymethylcysteine phenylthiohydantoin derivatives, which had low yields).

The gas-phase sequencer was operated with a double-cleavage double-extraction programme without vacuum as recommended by the manufacturers. Polybrene (1 mg) was added to the sintered-glass-fibre sample disc, which was then subjected to a 5 h cycle of washes before the sample was introduced. The Applied Biosystems sequencer automatically converted the thiazolinones into thiohydantoin derivatives by reaction with trifluoroacetic acid, and then delivered each sample into a Waters low-volume insert vial. The samples were dried in a Speedvac centrifuge for 10 min at room temperature. Acetate buffer, pH 5.0 (15 μl), and acetonitrile (15 μl) were then added at least 15 min before h.p.l.c. analysis as before.

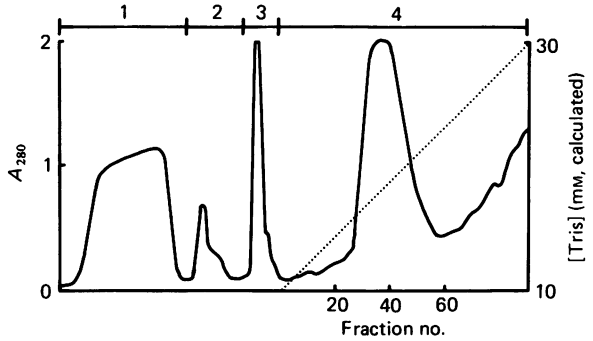


Fig. 1. Affinity elution of enolase from CM-cellulose

Partially purified enolase (total protein 4.6 g) in 10 mM-Tris/1 mM-magnesium acetate/0.2 mM-Na_2 EDTA adjusted to pH 6.7 with 1 M-Mops (final volume 500 ml) was loaded on to a column (5 cm × 10 cm) equilibrated with the same buffer. Buffers were applied as shown: buffer 1, 10 mM-Tris/1 mM-magnesium acetate/0.2 mM-Na_2EDTA adjusted to pH 6.7 with Mops; buffer 2, 10 mM-Tris/1 mM-magnesium acetate/0.5 mM-Na_2EDTA adjusted to pH 6.9 with Mops; buffer 3, 10 mM-Tris/1 mM-magnesium acetate/0.5 mM-phosphoenolpyruvate adjusted to pH 6.9 with Mops; buffer 4, a gradient consisting of 500 ml of buffer 3 plus 500 ml of 300 mM-Tris/1 mM-magnesium acetate/0.5 mM-phosphoenolpyruvate adjusted to pH 7.2 with Mops. The fraction size was 12 ml and the flow rate approx. 100 ml/h. The column was maintained strictly at 15–18 °C. ·····, Concn. of Tris; ——, A_{280}. Enolase (560 mg; specific activity 78 μmol/min per mg) was eluted in fractions 30–50.

RESULTS

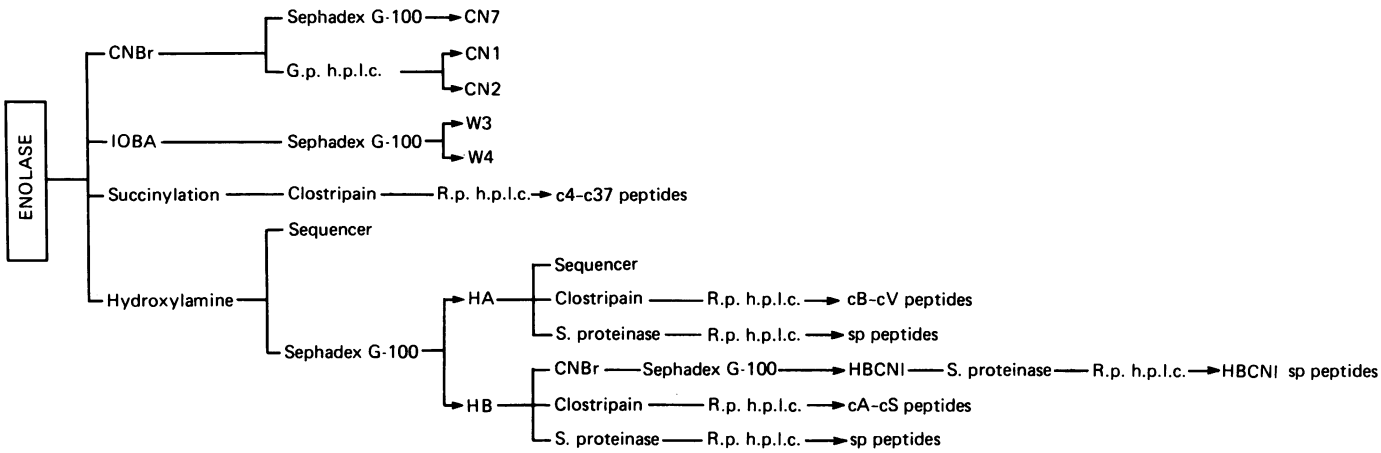
Purification and characterization of chicken enolase

An affinity-elution purification procedure modified from that of Scopes (1977*a*,*b*) was found to be an efficient

Table 1. Amino acid compositions of enolase and its fragments.

The results are expressed as mol of residue per mol of enolase subunit or per mol of fragment. For enolase and fragments HA and HB, the values for serine and threonine are corrected by extrapolation to zero time, and the values for hydrophobic residues were taken from the 96 h hydrolysis. Tryptophan was determined after hydrolysis with 3 M-mercaptoethanesulphonic acid for 96 h. Other values are averages of the three different times of hydrolysis. For the other fragments the values are from 20 h hydrolysis and are uncorrected. The values in parentheses are those found in the sequence. Methionine was detected as homoserine and/or homoserine lactone in the CNBr-cleavage peptides CN1, CN2 and CN7, and was not quantified. Abbreviations: Cm-Cys, carboxymethylcysteine; N.D., not determined

	Amino acid composition (mol/mol)																					
Amino acid	Enolase		HA+HB	HA		HB		CN1		CN2		CN7		W3		W4		c35		c37	
Cm-Cys	5.7	(6)	5.4	5.4	(6)	0.0	(0)	0.0	(0)	0.0	(0)	1.6	(2)	3.2	(5)	1.6	(2)	0.6	(1)	0.0	(0)
Asp	46.6	(29)	48.2	33.9	(21)	14.4	(8)	8.6	(6)	8.1	(2)	3.5	(2)	15.1	(6)	4.9	(3)	9.2	(5)	5.7	(2)
Asn		(15)			(9)		(6)		(1)		(6)		(1)		(5)		(2)		(3)		(3)
Thr	17.0	(14)	15.5	9.8	(8)	5.7	(6)	3.9	(4)	1.9	(2)	3.7	(3)	6.7	(5)	3.5	(3)	1.8	(2)	2.0	(2)
Ser	17.4	(21)	17.0	9.4	(13)	7.6	(8)	5.1	(5)	3.7	(4)	2.5	(3)	4.1	(7)	2.7	(3)	1.7	(2)	2.8	(4)
Glu	44.1	(30)	46.3	32.0	(18)	14.3	(12)	11.4	(9)	3.5	(3)	7.2	(4)	17.9	(8)	10.0	(6)	6.1	(5)	8.4	(7)
Gln		(14)			(12)		(2)		(2)		(0)		(3)		(8)		(3)		(1)		(1)
Pro	16.2	(16)	15.8	9.9	(10)	5.9	(6)	3.6	(3)	3.2	(3)	1.7	(1)	3.3	(3)	2.2	(2)	0.8	(1)	2.0	(2)
Gly	38.8	(41)	42.5	28.2	(27)	14.3	(14)	8.8	(8)	9.3	(9)	4.0	(4)	12.7	(11)	7.3	(7)	9.0	(8)	8.3	(8)
Ala	48.0	(48)	50.0	32.6	(31)	17.4	(17)	9.4	(8)	11.1	(11)	3.1	(3)	15.6	(14)	7.8	(7)	10.3	(10)	8.5	(8)
Val	34.1	(36)	32.6	19.0	(22)	13.6	(14)	8.4	(9)	4.9	(5)	1.7	(3)	9.1	(11)	3.5	(4)	5.9	(7)	8.2	(9)
Met	6.4	(7)	6.8	5.7	(6)	1.1	(1)	+	(1)	+	(1)	+	(1)	1.2	(2)	1.8	(2)	0.8	(1)	0.6	(1)
Ile	26.9	(29)	27.7	14.3	(15)	13.4	(14)	7.2	(10)	3.7	(4)	2.1	(2)	6.7	(7)	2.7	(3)	3.9	(4)	6.7	(7)
Leu	33.7	(33)	31.6	18.6	(20)	13.0	(13)	7.1	(7)	6.6	(7)	3.6	(4)	8.9	(10)	5.1	(5)	5.0	(5)	5.9	(6)
Tyr	8.5	(8)	5.7	4.8	(7)	0.9	(1)	0.0	(0)	0.8	(1)	0.9	(1)	0.4	(1)	0.3	(1)	2.1	(3)	0.8	(1)
Phe	15.5	(15)	15.6	11.7	(11)	3.9	(4)	2.6	(2)	2.0	(2)	1.3	(1)	4.0	(4)	3.0	(3)	2.3	(2)	2.2	(2)
Lys	37.8	(33)	34.6	20.2	(18)	14.4	(15)	9.6	(11)	4.2	(4)	1.8	(1)	9.0	(9)	6.2	(6)	7.5	(7)	10.5	(10)
His	16.1	(16)	17.1	9.8	(9)	7.3	(7)	4.3	(5)	3.2	(3)	0.4	(1)	4.5	(5)	0.8	(1)	1.8	(2)	2.6	(2)
Arg	17.2	(19)	17.6	11.8	(13)	5.8	(6)	5.0	(5)	0.8	(1)	2.9	(3)	6.6	(8)	5.6	(6)	0.9	(1)	0.8	(1)
Trp	3.0	(3)	(3)	N.D.	(3)	N.D.	(0)	N.D.	(0)	N.D.	(0)	N.D.	(0)	N.D.	(1)	N.D.	(0)	N.D.	(0)	N.D.	(0)
Total	433		433	280		153		96		68		43		130		69		70		76	



Scheme 1. Fragmentation of enolase

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

and specific method for purifying chicken skeletal-muscle enolase. A yield of approx. 60% (12 μmol of subunit from 650 g of muscle) was obtained in 3 days. The procedure involved initial partial purification by fractional precipitation with $(NH_4)_2SO_4$ and then elution from CM-cellulose in the presence of phosphoenolpyruvate (Fig. 1). The temperature of this affinity-elution step was kept strictly at 15–18 °C. The enzyme was homogeneous by polyacrylamide-gel electrophoresis (both with and without SDS), and had a specific activity of 1.3 μkat/mg at 20 °C. Enolase could be eluted from the CM-cellulose in the absence of phosphoenolpyruvate by a gradient of pH and ionic strength alone, but under these conditions a higher pH was required, and the enolase was contaminated with pyruvate kinase. The amino acid composition of the purified enolase is given in Table 1. The *N*-terminus of enolase is blocked, as no *N*-terminal residue could be detected when the enolase was subjected to Edman degradation in the liquid-phase sequencer.

Sequencing strategy and CNBr cleavage

Fragments of enolase were generated by cleavage with CNBr, *o*-iodosobenzoic acid, hydroxylamine and proteolytic enzymes as outlined in Scheme 1. Enolase has seven methionine residues, and our initial strategy was to isolate the CNBr-cleavage fragments as a first step in determining the sequence. This proved to be remarkably unproductive, and only fragment CN7 (43 residues) was readily obtained in good yield (Fig. 2). Examination by SDS/polyacrylamide-gel electrophoresis of the other peaks from the gel-filtration column showed that they were complex mixtures of partially cleaved and aggregated fragments. Several cleavage reaction conditions and a variety of separation methods were used in an attempt to purify the other CNBr-cleavage fragments. Fragments CN1 and CN2 could be obtained relatively pure although in low yield by gel-permeation h.p.l.c. No other CNBr-cleavage fragments were obtained in useful amounts, and it is now apparent that there are at least two properties of enolase that render it unsuitable for CNBr cleavage. A major difficulty is the presence of an

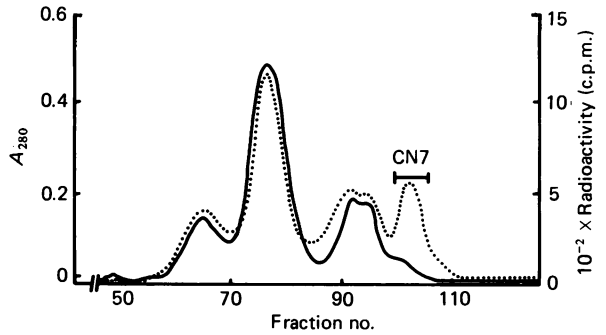


Fig. 2. Gel filtration of CNBr-cleavage fragments of enolase

Reduced and carboxymethylated enolase (90 mg) was dissolved in 9 ml of 70% (v/v) formic acid, and 20 mg of CNBr was added. The mixture was left for 24 h at room temperature, and then diluted 10–20-fold with water and freeze-dried. The sample was dissolved in 2.5 ml of 50% (v/v) formic acid, loaded on to the column and eluted with 50% (v/v) formic acid. The flow rate was 8 ml/h and 4 ml fractions were collected. ·····, Radioactivity; ——, A_{280}. Fractions 100–106 containing peptide CN7 were pooled.

unusually large number of acid-labile peptide bonds, which are susceptible to hydrolysis under the acid conditions used for CNBr cleavage. Our sequence results (see Fig. 7) show that chicken muscle enolase has three Asp-Pro sequences (residues 16–17, 265–266 and 293–294), with a peptide bond known to be particularly labile to relatively mild acid conditions (Landon, 1977). Another major problem was the tenacious aggregation of some of the CNBr-cleavage fragments even in the presence of 50% formic acid, 2 M-guanidinium chloride or 8 M-urea. A similar problem of aggregation of CNBr-cleavage fragments was encountered in the sequence determination of yeast enolase (Chin *et al.*, 1981*a*), although only two of the five methionine residues occupy equivalent positions (see Fig. 8).

The amino acid compositions of fragments CN1, CN2 and CN7 are given in Table 1. Fragment CN1 has a

blocked *N*-terminus and corresponds to the *N*-terminal 96 residues of enolase. Fragment CN2 corresponds to residues 97–164, and the sequence of its *N*-terminal 34 residues was determined (initial yield 2 nmol; repetitive yield 98%). *N*-Terminal sequencing of fragment CN7 yielded the sequence of 30 residues (initial yield 16 nmol; repetitive yield 90%). Fragment CN7 spans residues 368–410. The results of the sequence analysis are shown in Fig. 7.

o-Iodosobenzoic acid cleavage

o-Iodosobenzoic acid has been shown to be a highly specific reagent, cleaving peptide bonds *C*-terminal to tryptophan residues (Mahoney & Hermodson, 1979). However, acid conditions (80% acetic acid) are necessary for the cleavage reaction to take place. The suggested reaction conditions of 24 h at room temperature were modified to incubation for 6 h at 4 °C to minimize cleavage of acid-labile peptide bonds. Little or no acid cleavage was observed under these conditions, although complete cleavage at tryptophan residues was also not obtained. In fact this was an advantage, as fragment W3, a partial cleavage product corresponding to residues 304–433, could easily be separated from fragment W4 (residues 365–433) on Sephadex G-100 (Fig. 3). If complete cleavage had occurred, the two fragments, with 61 and 69 residues, would have been difficult to separate.

The amino acid compositions of fragments W3 and W4 are given in Table 1. A potential disadvantage of *o*-iodosobenzoic acid cleavage is the modification of tyrosine residues by contaminating *o*-iodoxybenzoic acid. It can be seen that very poor recovery of tyrosine was observed, although the *o*-iodosobenzoic acid was pre-incubated with *p*-cresol as recommended by Mahoney & Hermodson (1981). Fragment W3 was sequenced with an initial yield of 27 nmol and a repetitive yield of 92%, enabling about 30 residues to be identified. Sequence analysis of fragment W4 gave an initial yield of 13 nmol and a repetitive yield of 91%, and identified 33 residues. The sequencing results are shown in Fig. 7. The reason for the rather poor repetitive yields is not known, although it is notable that we have observed similar results for *o*-iodosobenzoic acid-cleavage fragments derived from aldolase (Freemont *et al.*, 1984).

Clostripain digestion

Clostripain has a preference for cleaving peptide bonds *C*-terminal to arginine residues (Mitchell & Hartington, 1971), although in our hands we observed significant cleavage *C*-terminal to lysine residues (see the results of the clostripain digestion of hydroxylamine-cleavage fragments HA and HB). The enolase was succinylated before treatment with clostripain to restrict digestion to arginine residues. Succinylation also served to render the carboxymethylated enolase soluble in the buffer used for digestion, and to minimize the aggregation of large peptides during separation. The clostripain-digest peptides were separated by h.p.l.c. on a C_{18} μBondapak reverse-phase column (Fig. 4). The peptide mixtures in peaks 23 and 27 were resolved by h.p.l.c. on a phenyl μBondapak reverse-phase column (the inset in Fig. 4 shows the separation of peak 23 peptides). The method was successful for purifying peptides as large as 69 residues (peptide c35) or 76 residues (peptide c37), although the two peptides in peak 30 (45 and 50 residues) could not be resolved on either C_{18} μBondapak or phenyl

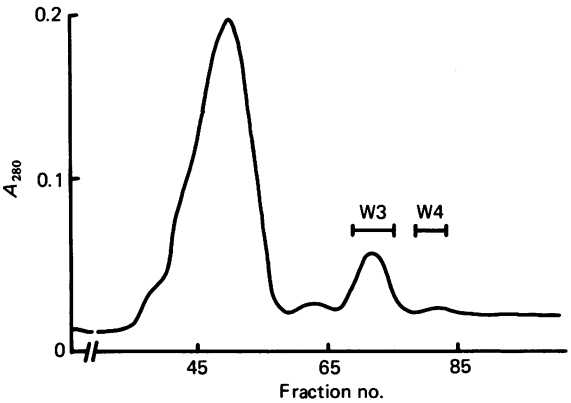


Fig. 3. Gel filtration of *o*-iodosobenzoic acid-cleavage fragments of enolase

Reduced and carboxymethylated enolase (1 μmol of subunit, 45 mg) was dissolved in 4.5 ml of 80% (v/v) acetic acid/4 M-guanidinium chloride, and 90 mg of *o*-iodosobenzoic acid (that had been preincubated with *p*-cresol) was added. The mixture was incubated for 6 h at 4 °C in the dark, desalted on Sephadex G-25 eluted with 10% formic acid, and freeze-dried. The sample was dissolved in 2 ml of 6 M-guanidinium chloride, diluted to 6 ml with water and loaded on to a column (2.5 cm × 170 cm) of Sephadex G-100. Elution was with 2 M-guanidinium chloride with a flow rate of 7.5 ml/h. The fraction size was 6 ml. ——, A_{280}. Fractions 69–75 and 78–82 containing peptides W3 and W4 were pooled.

μBondapak columns. These two peptides were successfully sequenced as a mixture. Peptide c30a had an initial yield of 34 nmol and peptide c30b one of 12 nmol. The quantitative identification of the phenylthiohydantoin derivatives at each cycle of Edman degradation meant that the two different sequences could be followed unambiguously. In a similar way, peptides c4a and c4b and peptides c13a and c13b were sequenced as mixtures.

The amino acid compositions of the two largest peptides, c35 and c37, are given in Table 1. The other peptides ranged in size from three to 25 residues, and their amino acid compositions agreed closely with the residues found by sequencing (Russell, 1984). The results of sequence analysis are given in Fig. 7.

Hydroxylamine cleavage

Cleavage of enolase with alkaline hydroxylamine generated two fragments, HA and HB, having M_r values of 30000 and 15000 respectively. The cleavage reaction was done for 3 h, and yielded about equal quantities of uncleaved enolase, fragment HA and fragment HB. Longer reaction times resulted in the appearance of an additional smaller cleavage fragment that was presumed to be derived from a minor cleavage at peptide bonds of the Asn–Xaa or Xaa–Pro type (Bornstein & Balian, 1977).

The *N*-terminus of enolase is blocked, and thus *N*-terminal sequence analysis of the unfractionated hydroxylamine-cleavage mixture would be expected to yield only a single sequence corresponding to the *C*-terminal fragment. The cleavage mixture from the 3 h cleavage procedure was carboxymethylated, desalted and sequenced. As expected, only one sequence was obtained, corresponding to the *N*-terminal 36 residues (see Fig. 7)

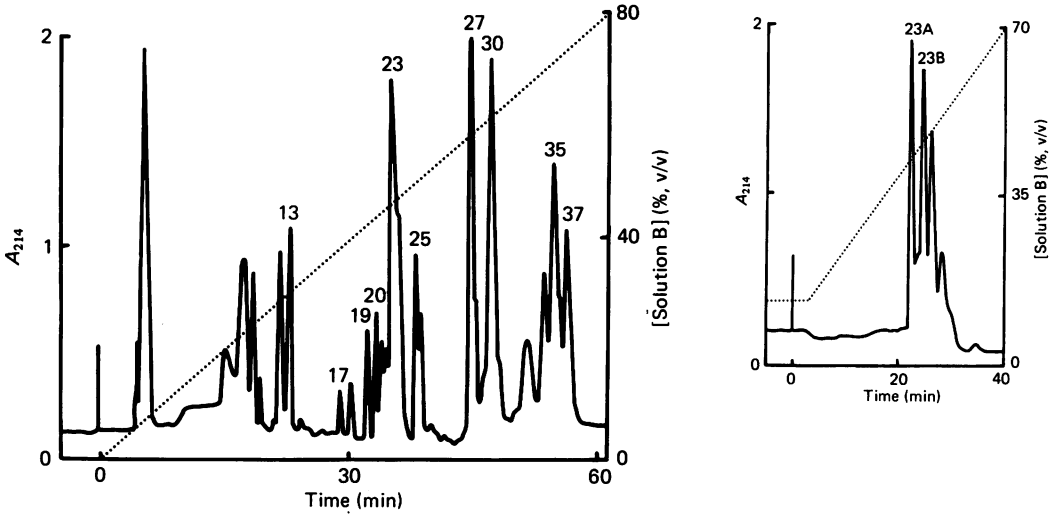


Fig. 4. Separation of clostripain-digest peptides from enolase by h.p.l.c.

Reduced, carboxymethylated and succinylated enolase (500 nmol) was dissolved in 12 ml of 75 mM-sodium phosphate buffer, pH 7.6, plus 12 ml of 7.5 mM-dithiothreitol, and digested with clostripain (4.5 mg) that had been 'activated' by preincubation in 1 mM-calcium acetate/2 mM-dithiothreitol overnight. Digestion was for 1 h at room temperature and 4 h at 37 °C, and was stopped by freeze-drying. The digest was dissolved in 2 ml of 0.1% (v/v) trifluoroacetic acid, and peptides in a 0.1 ml sample (25 nmol) were separated on a C_{18} μBondapak column as described in the text. ·····, Concn. of solution B; ——, A_{214}. Fractions corresponding to each major peak were collected. The inset shows the separation of peptides in peak 23 on a phenyl μBondapak column.

of the larger, *C*-terminal, fragment HA (residues 154-433). The initial yield was 60 nmol and the repetitive yield was 97%.

The two hydroxylamine-cleavage fragments were purified by gel filtration on Sephadex G-100 in 2 M-guanidinium chloride (Fig. 5). Attempts to separate the fragments in volatile solvents were unsuccessful: the fragments were insoluble in non-acidic solvents, and although soluble in 1% acetic acid or 10% formic acid were found to be aggregated. The amino acid compositions of purified fragments HA and HB are given in Table 1. It can be seen that the sum of the compositions of the two fragments corresponds to the composition of whole enolase.

A substantial portion of the sequence of enolase was established by determining the sequences of subpeptides of fragments HA and HB. The smaller peptides that resulted from subdigestion by clostripain or staphylococcal proteinase were relatively soluble and tended not to aggregate, and were thus readily separated by reverse-phase h.p.l.c. The determination of the sequences of peptides derived from fragments HA and HB are described in the next two subsections.

Peptides derived from fragment HB

Fragment HB (500 nmol) was digested with clostripain as described in Fig. 4 for the digestion of whole enolase. The peptides were separated on a C_{18} μBondapak reverse-phase column in a manner similar to that described in Fig. 4, except that a linear gradient of 0–60% organic solvent over 30 min was sufficient to separate most of the peptides (Russell, 1984). Ten peptides, ranging in size from three to 22 residues, were isolated, and the sequences of peptides cH, cJ, cK, cQ and cS were determined. The initial yields were about 20 nmol, and the complete sequence of each peptide was determined without difficulty except for peptide cS. In this case a

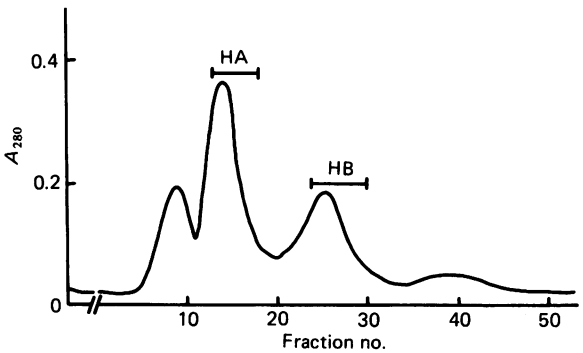


Fig. 5. Gel filtration of hydroxylamine-cleavage fragments of enolase

Enolase (3 μmol of subunit, 135 mg) was dissolved in 30 ml of 6 M-guanidinium chloride containing 300 μmol of hydroxylamine hydrochloride that had been adjusted to pH 9 with saturated LiOH. The mixture was kept at 45 °C for 3 h, and the pH was maintained at 9 with LiOH. The reaction was stopped by the addition of concentrated formic acid to give a pH of 2–3. The mixture was immediately desalted on Sephadex G-25 eluted with 10% formic acid, and freeze-dried. The sample was reduced and carboxymethylated, desalted and freeze-dried. It was then dissolved in 2 ml of 6 M-guanidinium chloride, diluted to 6 ml with water and loaded on to a column (2.5 cm × 170 cm) of Sephadex G-100. Elution was with 2 M-guanidinium chloride with a flow rate of 7.5 ml/h, and the fraction size was 6 ml. ——, A_{280}. Fractions 13–18 and 24–30 containing fragments HA and HB were pooled.

sequencer malfunction halted the sequence determination at residue 8. Automatic sequencing of the peptide designated cL gave two sequences in similar yield (22 and 23 nmol). Both peptides correspond to previously determined sequences, and could thus be distinguished.

The results of sequence analysis are given in Fig. 7. Peptides cA, cF and cG were not sequenced as they were small peptides (three or four residues) derived from regions of known sequence. The amino acid compositions of the clostripain-digest peptides agreed closely with the residues found by sequencing (Russell, 1984). Under the conditions used, clostripain was by no means specific for cleavage at arginine residues, and in fact seven of the ten peptides isolated resulted from cleavage at lysine residues.

Staphylococcal-proteinase digestion of fragment HB (300 nmol) was done under conditions specific for cleaving peptide bonds *C*-terminal to glutamic acid residues (Drapeau *et al.*, 1972). Staphylococcal-proteinase digestion of fragment HBCN1 (150 nmol) was also done. (This fragment was obtained by CNBr cleavage of fragment HB followed by gel filtration on Sephadex G-100 in 2 M-guanidinium chloride by a procedure similar to that described in Fig. 2.)

The staphylococcal-proteinase-digest peptides from fragment HB were purified by reverse-phase h.p.l.c. on a C_{18} μBondapak column, with a linear gradient of 0–60% organic solvent over 30 min. Thirteen peptides were isolated (Russell, 1984), and six (peptides sp19, sp20, sp22, sp23, sp25 and sp32) were completely sequenced with high initial yields (10–70 nmol). A seventh peptide (sp27) was sequenced with a much lower initial yield (2 mol), and was found to correspond to residues 210–224, presumably indicating that fragment HB was contaminated with a small amount of fragment HA. The results of sequence analysis are given in Fig. 7. Peptides sp3, sp4, sp10 and sp35 were not sequenced, and were placed by comparing their amino acid compositions with known sequences. Peptide sp14 was found to have a blocked *N*-terminus, and the sequence was determined by fast-atom-bombardment mass spectrometry (B. Gibson, D. Daley & D. H. Williams, unpublished work). The sequence of peptide sp13 was also determined by mass spectrometry. Generally there was good agreement between the amino acid compositions of the peptides and their sequences (Russell, 1984). There were two exceptions. The *N*-terminal peptide, sp14, had only 1.3 mol of isoleucine residues/mol of peptide, whereas two residues were found in the sequence. A longer hydrolysis time of 72 h gave a value of 1.5 mol of isoleucine residues/mol, indicating that the low value in the composition was probably a result of incomplete hydrolysis of the peptide bond adjacent to a hydrophobic amino acid residue. The other exception is peptide sp32, which had low values of serine and lysine. These residues were identified unambiguously by sequencing, and it is not clear why the composition values were low.

The peptides from staphylococcal-proteinase digestion of fragment HBCN1 were also purified by reverse-phase h.p.l.c. with a C_{18} μBondapak column. A linear gradient of 0–50% propan-2-ol over 20 min allowed the separation of five peptides (Russell, 1984). The peptides were placed by comparing their compositions with known sequences (see Fig. 7). Once again there was good correlation between the sequences and compositions (Russell, 1984). As noted before for peptide sp14, the *N*-terminal peptide HBCN1spH had a low value for isoleucine (1.8 mol of residues/mol compared with three residues found by sequencing).

Under the digestion conditions used there was no evidence that staphylococcal proteinase cleaved significantly adjacent to aspartic acid residues. However, a relatively unusual cleavage *C*-terminal to glutamine residues was observed for both peptide sp10 and the corresponding peptide from the HBCN1 digest, HBCN1spG. It is known that sequences with more than one glutamic acid residue can alter staphylococcal-proteinase specificity (Wootton *et al.*, 1975), and this may explain the susceptibility of glutamine in the Glu-Gln-Glu sequence of residues 85–87.

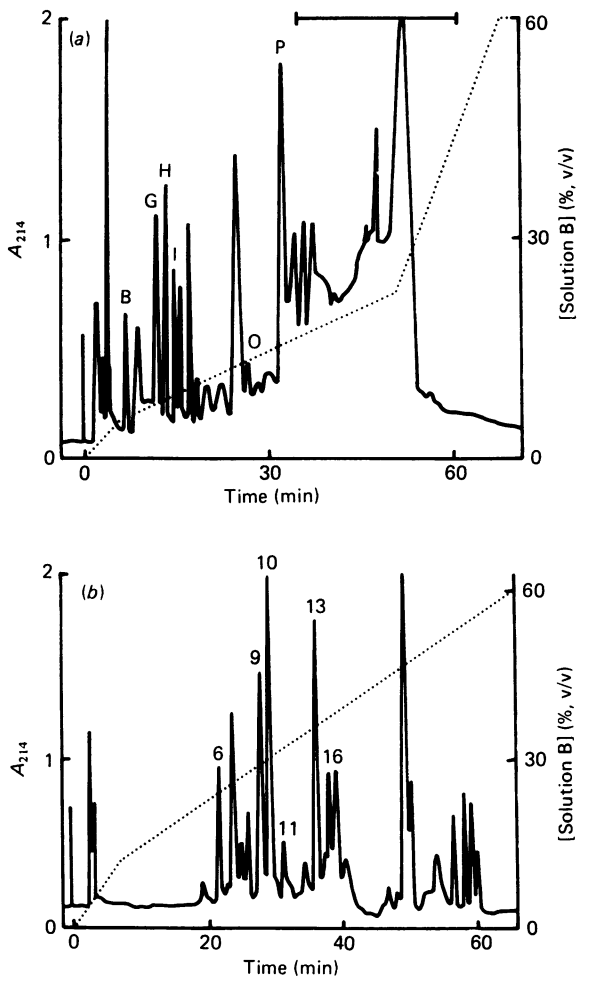


Fig. 6. Separation of staphylococcal-proteinase-digest peptides from fragment HA by h.p.l.c.

Carboxymethylated and succinylated fragment HA (200 nmol) was dissolved in 1 ml of 50 mM-NH_4HCO_3, pH 7.8, and digested with staphylococcal proteinase (2 × 0.06 mg) for 12–16 h at 37 °C. The digestion mixture was freeze-dried and dissolved in 0.6 ml of 0.1% (v/v) tirfluoroacetic acid, and the peptides in a 0.1 ml sample (30 nmol) were separated on a C_{18} μBondapak column (*a*) as described in the text. The peptides eluted after peak P were pooled, freeze-dried, dissolved in 0.1 ml of 0.1% trifluoroacetic acid and separated on a phenyl μBondapak column (*b*).

Peptides derived from fragment HA

Carboxymethylated fragment HA (200 nmol) was digested with clostripain as described in Fig. 4 for the digestion of whole enolase, except that the reaction was done for 1 h at room temperature and for 3 h at 37 °C. The peptides were dissolved in 0.1% NH_4HCO_3 and

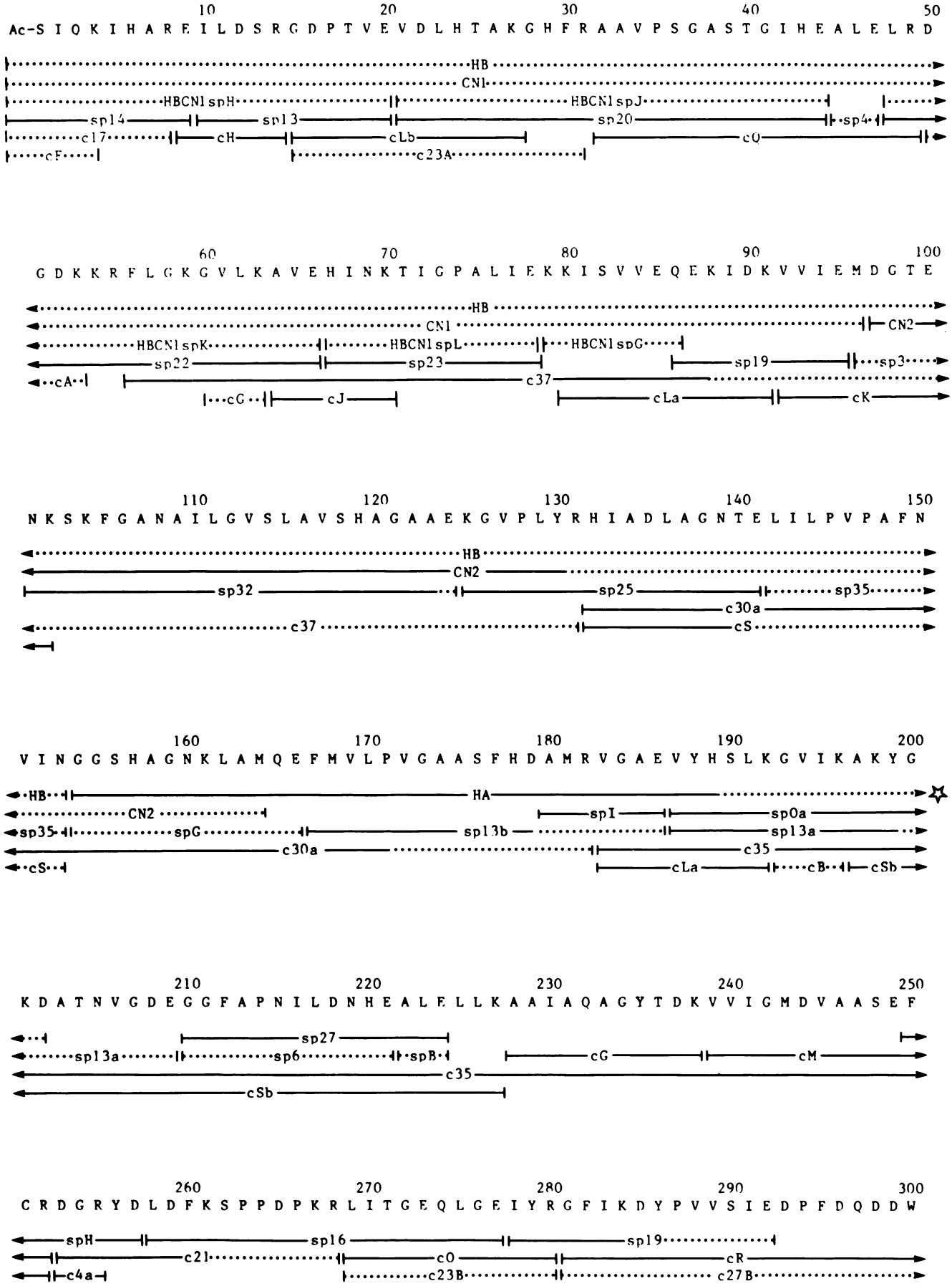


Fig. 7. For legend see facing page.

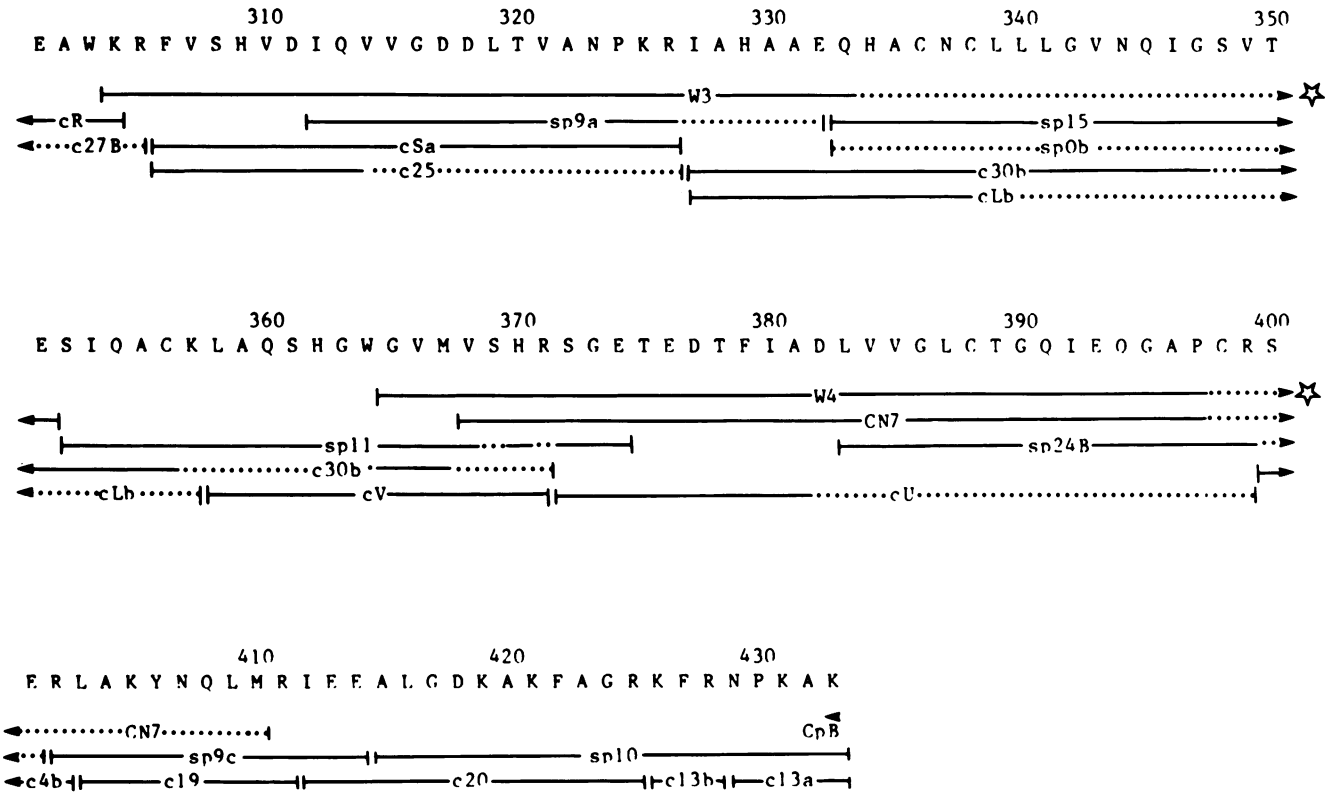


Fig. 7. Amino acid sequence of chick skeletal-muscle enolase

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

separated on a C_{18} μBondapak column with a linear gradient of 0–60% acetonitrile over 90 min. Eleven peptides ranging in size from four to 31 residues were isolated, and the complete sequences of peptides cG, cM, cO, cR and cV were determined with good initial yields (10–30 nmol). Only ten residues of peptide cV were identified, as the sequence of this region was already known. Automatic sequencing of peptide cL gave two sequences in similar yield (14 and 18 nmol), both corresponding to previously determined sequences. Similarly, peptide cS gave two sequences (11 and 12 nmol) that corresponded to previously sequenced regions. The results of sequence analysis are given in Fig. 7. Peptide cB was not sequenced, as it had only four residues and was from a region of known sequence. The amino acid compositions of the clostripain-digest peptides generally agreed well with the sequences (Russell, 1984), except that the value for carboxymethylcysteine in peptide cV was low (1.1 mol of residues/mol of peptide, whereas two residues were found in the sequence). A low value of this amino acid is not unusual in peptides purified by electrophoresis, and has been ascribed to partial oxidation of the sulphur (see, e.g., Allen, 1981). As in the case of fragment HB, cleavage at lysine residues occurred frequently.

Staphylococcal-proteinase digestion of carboxymethylated succinylated fragment HA (220 nmol) was done as described for fragment HB. The peptides were separated on C_{18} μBondapak and phenyl μBondapak columns (Fig. 6). The results of sequence analysis of 16 peptides are shown in Fig. 7. In addition, peptide spG was placed by composition and identification of the *N*-terminal amino acid; peptides sp6 and spB were placed by composition alone. Peptides sp9a and sp9c (13 and 8 nmol), peptides sp13a and sp13b (13 and 6 nmol) and peptides spOa and spOb (6 and 4 nmol) were successfully sequenced as mixtures. As before, there was generally good agreement between the results of amino acid analysis and sequencing (Russell, 1984). An exception was the absence of methionine in the composition of peptide spI, presumably because of oxidation of the sulphur.

Of the 18 peptide bonds of fragment HA cleaved by staphylococcal proteinase under the conditions used, 13 were at glutamic acid residues and five at aspartic acid residues.

DISCUSSION

The complete amino acid sequence of chicken skeletal-muscle enolase, comprising 433 residues, is summarized in Fig. 8. The subunit M_r value calculated from the sequence is 46797; this value is close to that of the yeast enzyme deduced from the sequence (M_r 46545) (Chin *et al.*, 1981*b*), or estimated by physical methods (M_r 88000–93000 for the dimer) (Mann *et al.*, 1970; Brewer *et al.*, 1978).

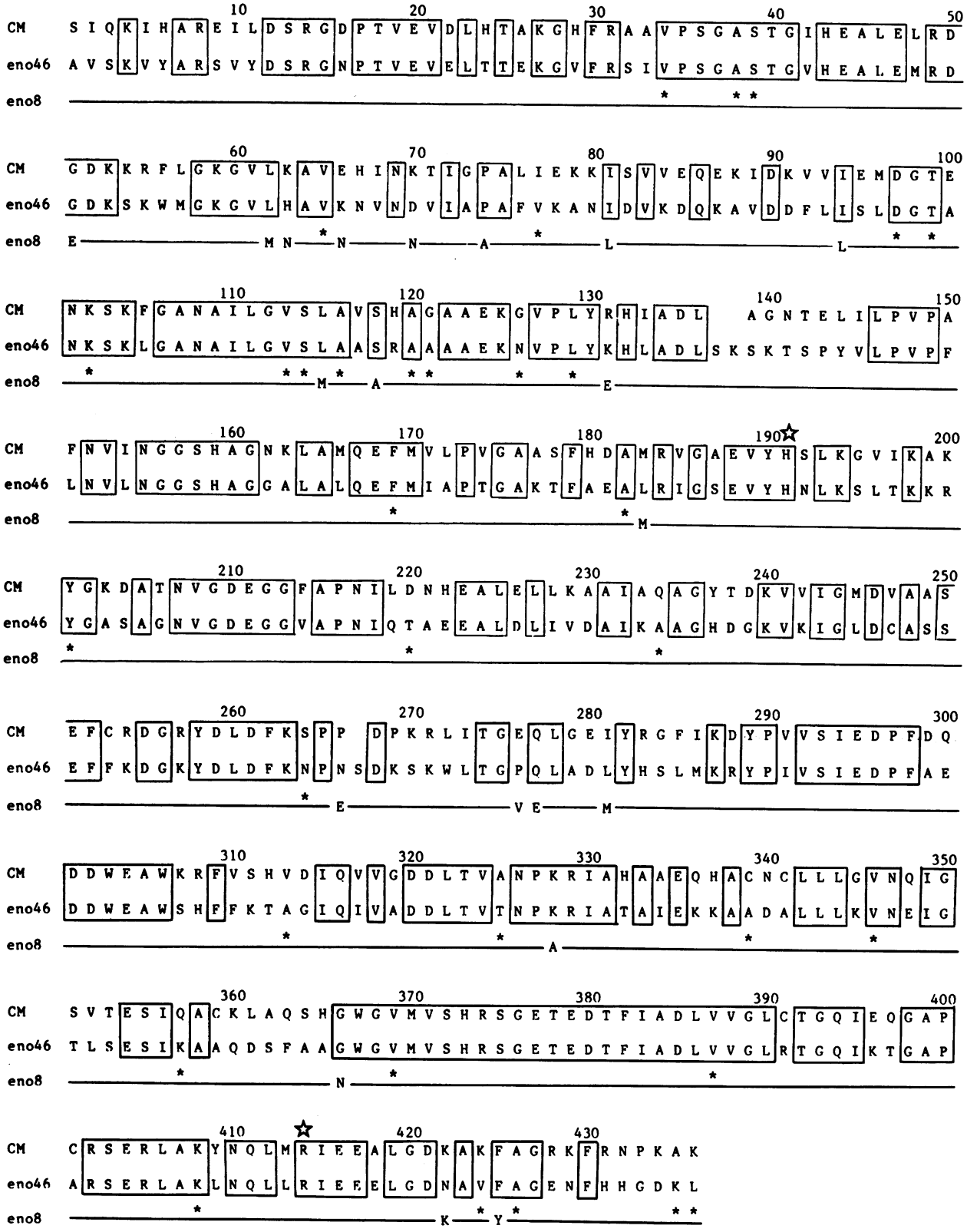


Fig. 8. Comparison of the sequence of chicken skeletal-muscle enolase with those of the yeast enolase isoenzymes

The sequences of the yeast enolases are taken from Holland *et al.* (1981). The residues are numbered in accordance with the yeast enolases. The sequences of the two yeast isoenzymes are identical as indicated by the continuous line except where a different amino acid is shown. The symbol * indicates that the codons differ at that position although the amino acids are identical. The boxed-in residues show the regions that are the same in the chicken and yeast eno 46 sequences. The symbol ☆ indicates the histidine and arginine residues implicated as being important for activity.

The nucleotide sequences of the two structural genes coding for the yeast enolase isoenzymes (eno 46 and eno 8) have been determined (Holland *et al.*, 1981), and the deduced amino acid sequences are compared with that of the chicken enzyme in Fig. 8. Protein sequence studies have also been done on the readily purified A isoenzyme of yeast. About 95% of the sequence of the A isoenzyme has been determined (Chin *et al.*, 1981*a*,*b*), and shows that it corresponds closely to that of eno 46 isoenzyme.

A comparison of the chicken and yeast enolase sequences shows that enolase is strongly conserved, with about 60% of the residues being identical. Enolase is thus evolving at about the same rate as cytochrome *c* (Dickerson & Geis, 1969). The chicken enzyme is slightly more similar to eno 46 isoenzyme than to eno 8 isoenzyme. As shown in Fig. 8, the two yeast isoenzymes differ at 20 positions. At 11 of these positions the chicken sequence is the same as that of eno 46 isoenzyme, whereas it is the same as that of eno 8 isoenzyme only twice. The chicken enolase is three residues shorter than the yeast enzymes, with a two-residue deletion at residues 137–138 and a single-residue deletion at position 267. The regions of least similarity between the chicken and yeast enzymes appear to correlate with portions of the polypeptide chain between elements of regular secondary structures (see the accompanying paper Sawyer *et al.*, 1986). They may thus correspond to inter-domain regions of the enzyme.

Chemical modification of yeast enolase with the photoactivated dye Rose Bengal resulted in the photo-oxidation of four histidine residues (Elliot & Brewer, 1979). However, in the presence of substrate and a high concentration of Mg^{2+} one histidine residue was protected, and a tryptic-digest peptide containing this residue was isolated. This peptide was shown to be His(Asx,Leu)Lys, and corresponds to residues 191–194 (Chin *et al.*, 1981*b*). The histidine residue is conserved in the chicken sequence, although the adjacent asparagine residue is replaced by a serine residue.

Treatment of yeast enolase with butanedione in borate buffer resulted in the modification of a single arginine residue, which could be protected by substrate (Elliott & Brewer, 1978). This residue was shown to be in a tryptic dipeptide, Leu-Arg. There are three Leu-Arg sequences in yeast enolase (at positions 183–184, 390–391 and 413–414) but in no case is the preceding residue a lysine or a arginine. The important arginine residue was tentatively concluded to be Arg-414, as this peptide was most likely to result from a chymotryptic-like cleavage at the preceding leucine residues (Chin *et al.*, 1981*b*). Arg-414 is conserved in the chicken enzyme, although the adjacent leucine residue is replaced by a methionine residue. It is most unlikely that Arg-391 can be the active-site arginine residue, as this residue is replaced by a cysteine residue in chicken enolase.

A carboxy group has been implicated as being important for activity (Rose & O'Connell, 1969; Russell & Fothergill, 1982), but it is not yet possible to identify the particular residue, as no labelled peptide has yet been isolated.

The determination of the sequence of chicken skeletal-muscle enolase is important for the interpretation of the X-ray-crystallographic structure of this enzyme. Crystals of the chicken enzyme have proved to be suitable for diffraction studies (H. C. Watson & P. J. Shaw, personal communication), and structural studies are under way. It will be of considerable interest to establish the detailed molecular structure of this important glycolytic enzyme.

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