THE ACTIVE CENTRE OF TRIOSE PHOSPHATE ISOMERASE FROM CHICKEN BREAST MUSCLE

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1. Introduction

Using the active-site-directed irreversible inhibitor bromohydroxy acetone phosphate (BHAP) Coulson et al. [1] isolated a hexapeptide from the active centre of chicken triose phosphate isomerase to which the inhibitor was bound covalently. Waley et al. [2] isolated a peptide of homologous sequence from the rabbit enzyme. Later de la Mare et al. [3] elucidated the residue to which the inhibitor moiety became attached and that to which it subsequently migrated. We have isolated from the chicken enzyme the tryptic peptide of fifteen residues that include the former hexapeptide sequence and have established its sequence by a combination of conventional and mass-spectrometric techniques. The sequence is identical to that derived for rabbit enzyme by Corran and Waley [4], but differs from those put forward, also for the rabbit enzyme, by Hartman [5, 6]. Preliminary results obtained by conventional techniques were reported by Offord et al. [7].

2. Experimental

2.1. *Materials*

Bromohydroxyacetone phosphate, triose phosphate, isomerase, pepsin, amino peptidase M and trypsin were obtained from the sources given in [3].

2.2. *Amino acid analysis and sequential Edman degradation* with detection of dansyl amino acids by polyamide thin-layer chromatography were performed as in [3].

2.3. *Mass spectra* of peptides after acetylation and permethylation [8] were run on an MS9 mass-spectrometer (AEI Scientific Apparatus) at a resolution (10% valley) of approx. 2000. The source was operated at 70 eV with a trap current of 100 μA. The sample was introduced in the all-glass, direct-insertion probe which can be heated up to 400°C independently of the source. The source block was maintained between 200–250°C.

2.4. *Electrophoretic mobilities* of peptides were measured as described by Offord [9]. The mobility (m) of aspartic acid at pH 6.5 is taken as -1.00.

2.5. *Digestion with pepsin.* Peptides were dissolved in 0.2 ml 7% (v/v) formic acid (pH 1.9) and a solution of pepsin in water was added to give a substrate: enzyme ratio of 25:1 (w/w). The reaction was terminated by freeze-drying after digestion for 4 hr at 37°C.

2.6. *Total enzymatic digestion with aminopeptidase M.* Peptide was digested under the conditions of de la Mare et al. [3]; these workers showed that the enzyme yielded free amino acids and the dipeptide Glu–Pro on digestion of the hexapeptide sequence of Coulson et al. [1]. As the procedure was employed here to estimate tryptophan content, prolidase was not used in case the manganese ion required attacked the tryptophan.

3. Results

3.1. *Conventional sequence studies on the tryptic peptide as isolated from chicken enzyme inhibited with bromohydroxyacetone phosphate*

Sixty milligrams of enzyme was reacted with inhibitor, carboxymethylated and digested with trypsin as described for enzyme treated with ^{14}C-labelled inhibitor [1].

After electrophoresis on No. 3MM paper of 6 mg digest at pH 6.5 on a 3 cm origin, the neutral band (which fluoresced under ultraviolet light) was cut and sewn to No. 3MM paper for electrophoresis at pH 8.9. This map was cut into four strips and these were stained respectively for tyrosine, with Pauly (histidine) reagent, by chlorination and with cadmium–ninhydin reagent followed by tryptophan staining. Two ninhydrin negative peptides were obtained, both tyrosine- and Pauly-positive, but only one (designated NN2) was tryptophan-positive. In its amino acid composition, end-group and electrophoretic mobilities as well as in its staining behaviour NN2 closely resembles the ^{14}C-labelled tryptic peptide of Coulson et al. [1] (J.D. Priddle, unpublished results). By the same criteria this peptide corresponds with the tryptic peptide T59a isolated during determination of the sequence of the native enzyme (A.J. Furth, J.D. Milman, J.D. Priddle and R.E. Offord, Biochem. J., in press).

NN2 was prepared from the remaining digest by pH 6.5 electrophoresis on No. 3MM paper at a loading of 0.8 mg/cm, followed by elution and rerunning on No. 1 paper at pH 8.9 on a 15 cm origin. It was eluted from paper with 0.25% (w/v) NH_4HCO_3. The peptide was digested with pepsin and on electrophoresis at pH 6.5 gave three peptides in significant yield: one acidic, one neutral and the other basic. These are designated NN2P2 (m = –0.31), NN2 NB(m = 0.0) and NN2 P3(m = +0.32) respectively.

NN2P2 stained for tyrosine and after purification by electrophoresis at pH 8.9 gave 150 nmole of peptide of composition $Glu_{1.1}Pro_{1.0}Ala_{1.0}Val_{0.9}Tyr_{0.9}$ with tryptophan seen on the short column of the amino acid analyser but not quantitated. This is clearly the hexapeptide Ala–Tyr–Glu–Pro–Val–Trp of Coulson et al. [1].

NN2NB and NN2P3 were eluted from paper (after over two years storage) and used for mass-spectrometry (see below).

3.2. *Sequence studies on the tryptic peptide isolated from the native enzyme*

The peptide T59a from 300 mg unmodified enzyme after carboxymethylation and tryptic digestion was purified after gel-filtration, and by electrophoreses at pH 6.5 and pH 8.9 as described above. Its amino acid composition was (96 hr hydrolysis) $Lys_{0.9}Thr_{1.1}Glu_{1.4}Pro_{1.4}Gly_{2.3}Ala_{2.4}Val_{2.75}Ile_{1.0}$ $Leu_{0.9}Tyr_{0.9}Trp_{+}$. The N-terminus was valine.

Twenty nanomoles were directly degraded by the dansyl–Edman technique giving Val–Val–Leu–Ala–Tyr, taking the sequence unambiguously into the hexapeptide sequence of Coulson et al. [1]. 130 nmole was digested with pepsin and three major peptides prepared by electrophoresis at pH 6.5: one acidic, m = –0.34 (T59aP1), one neutral, m = 0.0 (T59aNB1) and one basic, m = +0.30 (T59aP2). From its amino acid composition and end-group T59aP1 is equivalent to NN2P2.

Acid hydrolysis of T59a NB1 for 96 hr and amino–acid analysis gave the composition $Val_{2.0}Leu_{1.0}$ confirming the dansyl–Edman result on the intact material.

Sequential degradation of T59aP2 gave the sequence Ala–Ile–Gly–(Thr, Gly)Lys. Although it appeared more likely that the threonine residue preceded the second glycine, the dansyl–threonine was contaminated with a small amount of dansyl–glycine. The lysine residue was not detected but assigned from the electrophoretic mobility of the peptic peptide, the composition of the tryptic peptide and the expected specificity of the initial tryptic cleavage. Total enzymatic hydrolysis of 20 nmole T59a gave all the free amino acids expected except glutamic acid and proline (cf. [3]). The ratio of tryptophan to lysine was 0.64:1, implying that there may be only a single residue of tryptophan in T59a.

3.3. *Sequence studies using mass-spectrometry*

The mass-spectrum (fig. 1a) of NN2NB (about 150 nmole) after acetylation and permethylation confirms the sequence Val–Val–Leu, while that of NN2P3 (about 150 nmole, fig. 1b) indicates the sequence Ala–Ile–Gly–Thr–Gly–.

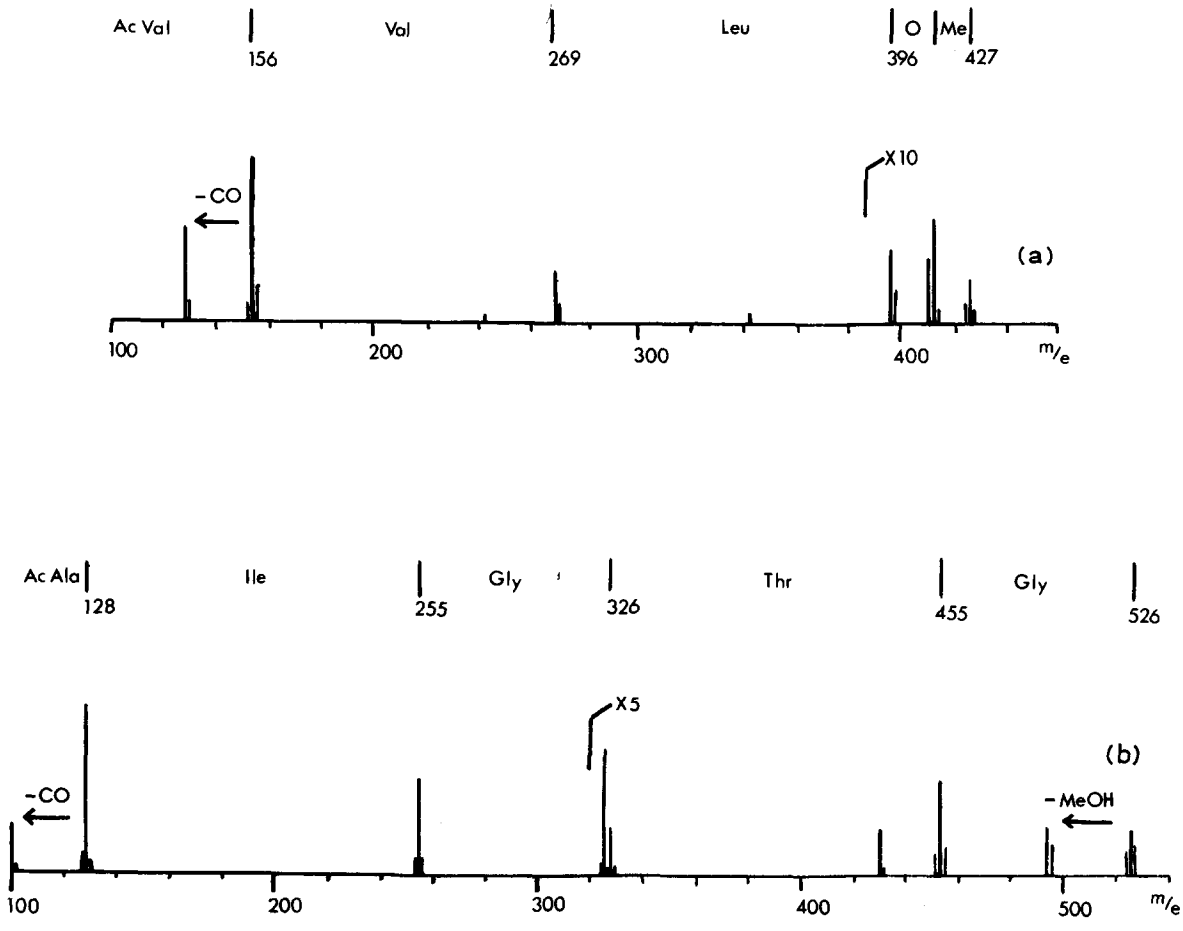

Fig. 1. The mass-spectra (above *m/e* 100) of the peptides NN2NB and NN2P3. (a) The mass spectrum of NN2NB. Probe temperature 100 °C, source temperature 250°C. The sequence ions show the order Val–Val–Leu and the molecular ion (*m/e* 427) indicates the C-terminal methyl ester. To simplify the figure, the normal amino acid abbreviations are used to represent the modified residues. Mass differences between sequence ions cannot distinguish leucine from isoleucine, but in the present cases prior amino acid analyses established the identity of the residues in question. (b) The mass spectrum of NN2P3. Probe temperature 170°C, source temperature 200°C. The sequence ions show the order Ala–Ile–Gly–Thr–Gly– while conventional techniques (for detail see the text) gave Ala–Ile–Gly(Thr,Gly)Lys.

4. Conclusion

We conclude that the amino acid sequence of the tryptic peptide that contains the active-site glutamyl residue is Val–Val–Leu–Ala–Tyr–Glu–Pro–Val–Trp–Ala–Ile–Gly–Thr–Gly–Lys. This is identical in sequence to the analogous peptide in the rabbit enzyme [4]. Studies on the active site of the rabbit enzyme have also been reported by Hartman [5, 6, 10–14]. One difference remains between our sequence and that most recently published by Hartman [6]. This is that the first residue of the peptide is given as tryptophan, rather than valine. Such a difference between the chicken and rabbit protein is not impossible (but see [4]). However, Hartman assigned tryptophan to this position on the basis of a negative result with the subtractive Edman degradation [15]. He observed that the amino acid composition of the peptide remained unaltered after a single cycle of the Edman procedure. It was assumed that this could only be explained if the first residue was tryptophan, since, because of its instability to acid hydrolysis, it would

make no contribution to the amino acid composition of the undegraded peptide.

If, however, the first residue is valine, as well as the second, the resulting Val–Val bond would be partially resistant to hydrolysis with 6N HCl. Since the peptides were hydrolysed for 21 hr [5, 6] about 50% of this dipeptide sequence would be expected to remain unhydrolysed. Hydrolysis of the undegraded peptide could therefore lead to the same composition as that for the peptide after the first Edman cycle. This would be despite any possible compensation that might have arisen as a result of normalising the composition to isoleucine [6], another residue that is often slowly liberated. We therefore conclude that Hartman has not yet unambiguously excluded the possibility of a third valine residue in the tryptic peptide or placed his second tryptophan.

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