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SEZIONE II

(Fisica, chimica, geologia, paleontologia e mineralogia)

Chimica biologica. — *The Primary Structure of Aspartate Aminotransferase from Pig Heart Muscle. Peptides Produced by Cleavage with Cyanogen Bromide and with Dilute Acid* (*). Nota di ROBIN HANFORD, HILARY J. DOONAN, SHAWN DOONAN, CHARLES A. VERNON, JOHN WALKER, FRANCESCO BOSSA, DONATELLA BARRA, MASSIMO CARLONI, PAOLO FASELLA e FRANCESCA RIVA, presentata (**) dal Socio A. ROSSI-FANELLI.

RIASSUNTO. — Vengono presentati i risultati della scissione con il bromuro di cianogeno dell'aspartato aminotransferasi carbossimetilata. È stato possibile isolare i peptidi risultanti dalla scissione avvenuta a livello di quattro dei sei residui di metionina presenti nella molecola. Inoltre sono stati ottenuti peptidi derivanti da una scissione a livello di residui di triptofano e dalla idrolisi acida parziale di legami aspartil-prolina. Sono stati eseguiti dei tentativi per sfruttare questa labilità in acido dei legami aspartil-prolina con una digestione controllata della proteina in acido diluito. I risultati hanno indicato una scissione preferenziale alle posizioni desiderate ma si è anche avuta un'estensiva idrolisi di altri legami peptidici.

INTRODUCTION

We have recently presented a primary structure for the cytoplasmic aspartate aminotransferase from pig heart muscle (Doonan *et al.*, 1974); the structure is shown in Table V. Similar results were obtained by Ovchinnikov *et. al.* (1973). Our results were obtained by the application of a wide variety of enzymic digests (Doonan *et al.*, 1972; Bossa *et. al.*, 1973 and accompanying papers) including digestion with a newly characterized protease with specificity for lysine (Doonan *et al.*, 1974). In view of the wide-spread success which has attended the cleavage of proteins with cyanogen bromide since the original work by Gross and Witkop (1961), we have attempted to apply this method to aspartate aminotransferase. The work was severely complicated due to cleavage of the molecule at tryptophan residues and to partial acid hydrolysis of aspartyl-proline bonds. The latter problem was also encountered by Langley and Smith (1971) in their work on glutamate dehydrogenase. We have made use of acid lability of aspartyl-proline bonds in a partial acid hydrolysis of the protein, the results of which are also presented here.

(*) This research was carried out in the Christopher Ingold Laboratories, Department of Chemistry, University College London, and in the Istituto di Chimica Biologica e Centro di Biologia Molecolare del Consiglio Nazionale delle Ricerche, Università di Roma.

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EXPERIMENTAL

MATERIALS.

Aspartate aminotransferase was purchased from Whatman Biochemicals Ltd., Maidstone, Kent, U.K.; this material is prepared essentially as described by Banks *et al.*, (1968). Cyanogen bromide was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.

METHODS.

General methods of protein modification, peptide purification, determination of amino acid compositions by the method of complete dansylation, digestion with carboxypeptidase A, N–terminal analysis and sequence determination were as previously described (Doonan *et al.*, 1972, Bossa *et al.*, 1973 and Supplementary Publications). Particular techniques used in the present work were as follows.

Cleavage with cyanogen bromide. In a typical cleavage reaction, Cm–Enzyme [1] (0.5 g) was dissolved in formic acid (70 % v/v, 50 ml). Cyanogen bromide (5.0 g) was added and the flask flushed with nitrogen and stoppered. The sample was incubated for 21 h at 37° after which water (250 ml) was added and the solution dried by lyophilization.

Purification of peptides from cleavage with cyanogen bromide. The peptide mixture was dissolved in acetic acid (50 % v/v, 28 ml) and fractionated on a column (3.3 × 100 cm) of Sephadex G–50 equilibrated in acetic acid (15% v/v). Components of separated fractions were further purified by conventional techniques of paper chromatography and high voltage paper electrophoresis or by ion exchange chromatography. For ion exchange chromatography on SP Sephadex C–25, the resin was equilibrated with acetic acid (30 % v/v) and the sample applied in the same solvent. When no more material was eluted with this solvent, a positive parabolic gradient of NaCl was applied to elute absorbed material. Fractions were desalted by passage through Sephadex G–50. Peptide mixtures which were insoluble in acetic acid solution were maleylated (Butler *et al.*, 1969) and then subjected to fractionation on QAE Sephadex A–25. The resin was equilibrated in NH_4OH (0.1 M) and the sample applied in this solvent. Again absorbed material was eluted by application of a parabolic salt gradient. Fractions were desalted by gel filtration and then demaleylated by incubation in acetic acid (1% v/v) for 6 h at 60° (Butler *et al.*, 1969).

(1) *Abbreviations*: Cm–Enzyme, carboxymethylated aspartate aminotransferase; Hse, homoserine; CmCys, carboxymethylcysteine.

Cleavage with dilute acid. Cm-enzyme (0.9 g) was suspended in formic acid (5 % v/v, 90 ml) and the suspension incubated at 70° for 27 h. The resulting solution was dried by rotary evaporation.

Purification of peptides from cleavage with dilute acid. The peptide mixture was maleylated before initial separation using Sephadex G-75 equilibrated in NH_4OH (0.01 M). Fractions from gel filtration were further purified by ion exchange chromatography on QAE Sephadex A-25 as described above, and finally demaleylated.

N-terminal analysis and amino acid analysis. The purity of peptides from the cyanogen bromide digest was established by the dansylation technique of Gray (1967). In the case of peptides from the digest with dilute acid, many peptides had the same N-terminal residue (proline) and hence this was not a good test of purity. Peptides with N-terminal proline were subjected to a cycle of the dansyl-Edman degradation (Hartley, 1970) and the appearance of a single new N-terminal residue was taken as proof of purity. Qualitative amino acid analyses were carried out by the method of complete dansylation (Doonan *et al.*, 1972) and were in all cases consistent with sequences obtained.

Amino acid sequence determinations. For peptides of up to about twenty amino acid residues, sequences were determined by direct application of the dansyl-Edman method (Hartley, 1970). Large peptides available in large amounts (ca. 10 mg) were subjected to digestion with trypsin and the fragments separated and sequenced. In the case of large fragments available in only small quantities, the mixture of peptides obtained by digestion with trypsin was sequenced without separation of the components. At each step several N-terminal residues were observed, depending on the number of peptides in the mixture, but it was usually possible to assign unambiguous sequences based on previous knowledge of the region of the protein from which the large fragment originated.

Redigestion of large fragments with trypsin. The peptide was dissolved or suspended in N-ethyl morpholine buffer (1-2 ml, 0.2 M, pH 7.5) containing trypsin (1 : 50 w/w, Sigma (London) Chemical Co, London S.W. 6, U.K., Type XI) and incubated at 37° for 2 h, after which the digest was dried by rotary evaporation.

RESULTS

Peptides produced by cleavage with cyanogen bromide. Sequence data for peptides isolated from the digest with cyanogen bromide are shown in Table I. Also shown are the stages of purification for each peptide and the positions of the residues in the complete structure of aspartate aminotransferase (Table V), The first 14 residues of peptide CN2 were determined using the dansyl-Edman method. The remainder of the material (ca. 20 mg) was redigested with trypsin and the resulting fragments isolated and sequenced (Table II).

TABLE I.

Peptides isolated after digestion of aspartate aminotransferase with cyanogen bromide.

E, electrophoresis at pH 3.5; I_C and I_A, ion exchange chromatography on SP Sephadex C–25 and QAE Sephadex A–25 respectively; C, chromatography; G, gel filtration on Sephadex G–50. Residues were identified by the dansyl-Edman method except for those marked which were liberated by treatment with carboxypeptidase A.

Peptide	Purification Stages	Sequence Data	Position in Final Structure
CN1	E	Pro–Asx	28–29
CN2	I_C, G	Val–Leu–Pro–Val–Val–Arg–Lys–Val–Glx–Glx–Arg–Ile–Ala–Asx–	49–121 (see Table II)
CN3	C, E	Tyr–Asx–Gly–Thr–Asx	123–127
CN4	E	Pro–Thr–Pro–Glx–Glx	200–204
CN5	E	Lys–Glx–Ile–Ala–Ser–Val–Hse	206–212
CN5a	I_A	Lys–Glx–Ile–Ala–.....Glu–Hse	206–287
CN6	E	Glx–Lys–Ile–Val–Arg–Val–Thr	288–294
CN7	E, C	Pro–Glx–Leu–Phe–His–Glx	313–318
CN8	I_C, G	Ala–Asx–Arg	327–
CN9	I_C, G	Leu–Leu–Pro–Ser–Gly–Arg–Ile–Asx–Hse	381–389

TABLE II.

Fragments isolated from a tryptic digest of peptide CN2.

In all cases, except for peptide CN2-d, the complete sequence was obtained by the dansyl-Edman method. Peptide CN2-d was isolated in insufficient yield to allow sequence determination.

Tryptic Peptide	Sequence	Position in Final Structure
CN2-a	Val-Leu-Pro-Val-Arg	49-54
CN2-b	Val-Glx-Glx-Arg	56-59
CN2-c	Ile-Ala-Asx-Asx-Ser-Ser-Leu-Asx-His-Glx-Tyr-Leu-Pro-Ile-Leu-Gly-Leu-Ala-Glx-Phe-Arg	60-80
CN2-d	Thr.....	81-
CN2-e	Leu-Ala-Leu-Gly-Asx-Asx-Ser-Pro-Ala-Leu-Glx-Glx-Lys	86-98
CN2-f	Val-Gly-Gly-Val-Glx-Ser-Leu-Gly-Gly-Thr-Gly-Ala-Leu-Arg	100-113
CN2-g	Ile-Gly-Ala-Glx-Phe-Leu-Ala-Arg	114-121
CN2-h	Free Lys + Free Arg	55, 99

Reference to Table V shows that the tryptic peptides cover the region of residues 49 to 121 of the final structure. The fragment containing residues 81–85 was not obtained in sufficient yield for sequence analysis but a peptide was obtained (CN2–d) which had N–terminal threonine.

In addition to peptide CN3, larger fragments were isolated in very low yield with the same N–terminal sequences but not terminating at the Asx residue. Insufficient material was obtained to characterize these fragments further. Peptide CN5–a was isolated from a portion of the digest which had been rendered soluble by maleylation. The N–terminal sequence was Lys–Glx–Ile–Ala– (cf. CN5) and on treatment with carboxypeptidase A homoserine and glutamic acid were liberated (identified by dansylation). Hence the peptide covered residues 206–287 of the final structure. Peptide CN8 was also obtained in too small quantity for complete characterization. The N–terminal sequence showed that it started at position 327 but qualitative amino acid analysis by complete dansylation indicated that it did not terminate at the next methionine residue in the polypeptide chain (residue 333).

Peptides produced by cleavage with dilute acid. Sequence data for the peptides isolated after partial acid hydrolysis of carboxymethylated aspartate aminotransferase are shown in Table III. Peptides A1a and A1b were subjected to digestion with trypsin and the fragments isolated and sequenced. The results are shown in Table IV. It is of interest that both peptides A1 a–b and A1 b–b contained an internal arginine residue; tryptic cleavage at this bond was presumably restricted by the adjacent glutamic and aspartic acid residues. Peptides A2, A3, A7 and A8 were isolated in very low yield, and only sufficient material was available to establish their N–terminal sequences. Consequently it was not possible to define at which residues in the final structure these peptides terminated.

A portion of each of the peptides A1d, A6a, A10 and A11 was subjected to the dansyl-Edman procedure in order to establish their N-terminal sequences. The remaining material was then digested with trypsin and the mixtures of fragments produced were sequenced without separation. For example, in the case of peptide A10, the N–terminal sequence was Pro–Lys–Glx–Val–. N–terminal analysis of the tryptic digest of peptide A10 showed Pro, Glx, His and Ile. After one cycle of Edman degradation the new N–terminal residues were Lys, Val, Ile and Asx. After two cycles the residues were Glx and Tyr, and so on. From the N–terminal sequence it was obvious that the peptide started at residue 367 and it follows from the specificity of trypsin that the residues seen on N–terminal analysis of the digestion mixture were Pro367, Glx369, His378 and Ile387. After one cycle of Edman degradation the new N–terminal residues were Lys368, Val370, Ile379 and Asx388, after two cycles the residues were Glx371 and Tyr380. In this way the C–terminus of the peptide was shown to be Asx388 and the remainder of the sequence in this region was confirmed. This indirect approach to sequencing of large

TABLE III.

Peptides isolated after digestion of aspartate aminotransferase with dilute acid.

All amino acid residues shown were determined by the dansyl–Edman technique. Peptides such as AId which are divided by vertical bars were subjected to tryptic digestion and the fragments sequenced without previous separation (see text). Alignment of the component fragments was by comparison with known sequences.

Peptide	Sequence Data	Position in Final Structure
AIa	Ala–Pro–Pro–Ser–Val–	1–62 (see Table IV)
AIb	Ala–Pro–Pro–Ser–Val–	1–27 (see Table IV)
AIc	Pro–Asx	28–29
AId	Pro–Arg+Lys+Val–Asx–Leu–Gly–Val–Gly–Ala–Tyr–Arg+Thr–Asx–Asx–CmCys–Glx–Pro–Trp–Val–Leu–Pro–Val–Val–Arg+Lys+Val–Glx–Glx–Arg+Ile–Ala–Asx	30–62
A2	Ser–Pro–Ala–Leu.....	92–
A3	Ile–Gly–Ala–Glu–Phe–Leu....	114–
A4	Lys–Asx	129–130
A5	Tyr–Trp	160–161
A6a	Pro–Thr–Pro–Glx–Glx–Trp–Lys+Glx–Ile–Ala–Ser–Val–Met–Lys+Arg+Arg+Phe	200–216
A6b	Pro–Thr–Pro–Glx–Glx–Trp	200–205
A6c	Pro–Thr–Pro	200–202
A7	Ser–Ile–Leu–Arg–Val–Leu–Ser.....	279–
A8	Pro–Pro–Ala–Glx–Gly....	298–
A9a	Pro–Glx–Leu–Phe–His–Glx–Trp–Thr–Gly–Asx–Val–Lys–Thr–Met–Ala–Asx	313–328
A9b	Pro–Glx–Leu–Phe–His–Glx–Trp–Thr–Gly–Asx	313–322
A10	Pro–Lys+Glx–Val–Glx–Tyr–Leu–Ile–Asx–Glx–Lys+His–Ile–Tyr–Leu–Leu–Pro–Ser–Gly–Arg+Ile–Asx	367–388
A11	Met–CmCys–Gly–Leu–Thr–Thr–Lys+Asx–Leu–Asx–Tyr–Val–Ala–Thr–Ser–Ile–His–Glx–Ala–Val–Thr–Lys+Ile–Glx	389–412

TABLE IV.

Fragments isolated from tryptic digests of peptides AIa and AIb

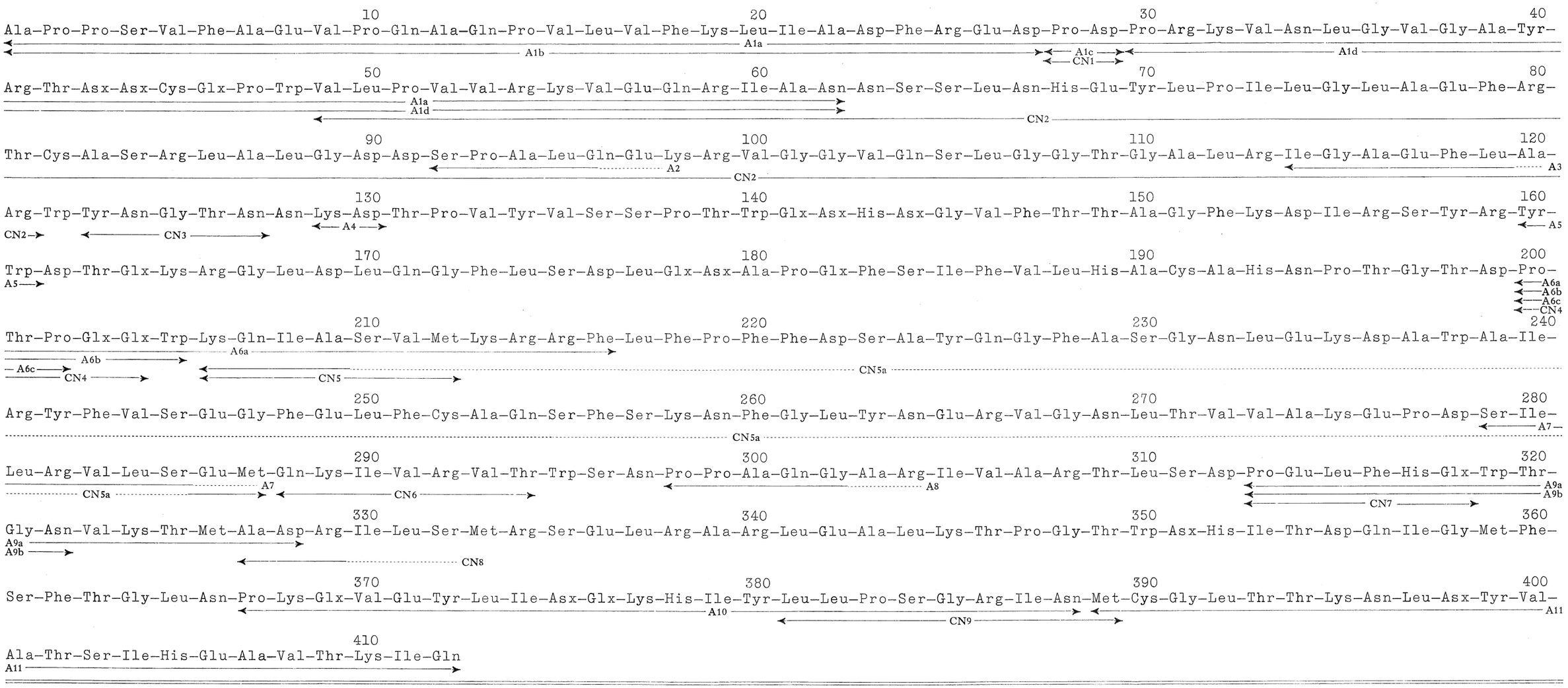
In all cases the sequences were determined by repeated application of the dansyl–Edman procedure.

Tryptic Peptide	Sequence	Position in Final Structure
AIa–a	Ala–Pro–Pro–Ser–Val–Phe–Ala–Glx–Val–Pro–Glx–Ala–Glx–Pro–Val–Leu–Val–Phe–Lys	1–19
AIa–b	Leu–Ile–Ala–Asx–Phe–Arg–Glx–Asx–Pro–Asx–Pro–Arg	20–31
AIa–c	Val–Asx–Leu–Gly–Val–Gly–Ala–Tyr–Arg	33–41
AIa–d	Thr–Asx–Asx–CmCys–Glx–Pro–Trp–Val–Leu–Pro–Val–Val–Arg	42–54
AIa–e	Lys–Val–Glx–Glx–Arg	55–59
AIa–f	Ile–Ala–Asx	60–62
AIb–a	As for AIa –a	1–19
AIb–b	Leu–Ile–Ala–Asx–Phe–Arg–Glx–Asx	20–27

TABLE V.

Primary structure of aspartate aminotransferase showing positions of the fragments isolated from the digests with cyanogen bromide and dilute acid.

The letters CN and A refer to cyanogen bromide and dilute acid fragments respectively. Solid lines are placed beneath residues which were identified directly and dashed (– – –) lines beneath residues which were assigned by comparison with known sequences. A dotted line (· · · ·) indicates that the C-terminus of the peptide was not identified.



fragments available in restricted quantity has been found to be very useful, but depends on extensive previous information about the structure of the molecule in the region from which the fragment is derived.

DISCUSSION

The positions of the fragments isolated after cleavage of aspartate aminotransferase with cyanogen bromide and dilute acid are marked on the structure shown in Table V. The structure is a previously published (Doonan *et al.*, 1974) except for further acid and amide assignments some of which are the results of our unpublished work while others (positions 162, 199, 297, 312 and 366) have been taken from the structure due to Ovchinnickov *et al.* (1973).

Digestion with cyanogen bromide met with only limited success in that the peptides which were fully characterized covered only about 25 % of the amino acid residues in the protein. The cleavage points observed are, however, of considerable interest. Definite evidence of cleavage was obtained for only four of the six methionine residues. Of these, cleavage at Met–212 was incomplete since fragment CN5–a contains this methionine residue at an internal position. Tentative evidence for cleavage at Met–359 was provided by the observation that the original digest contained a peptide with N–terminal phenylalanine; attempts to isolated this component were unsuccessful. No peptide was obtained originating from cleavage at Met–333; peptide CN 8 contained this residue internally. There have been several reports of failure of cyanogen bromide to cleave at Met–Thr or Met–Ser bonds (for example Langley and Smith, 1971; De Lange, 1970; Schroeder *et al.*, 1969). Schroeder and his coworkers (1969) attributed this to attack of the seryl or threonyl side chain oxygen on the iminolactone intermediate of the cyanogen bromide reaction with formation of a stable five-membered ring compound. This is unlikely to be the cause of failure to cleave at a Ser–Met bond since the corresponding cyclic product would contain a seven rather than a five-membered ring. Corradin and Harbury (1970) have proposed a more general mechanism for incomplete cleavage where hydrolysis of the iminolactone intermediate is partitioned between peptide bond fixion and opening of the iminolactone to produce homoserine but leaving the peptide chain intact; it is possible that the second route is the major or exclusive one in the case of Met–333.

In addition to cleavage at methionine residues, fragmentation at two other types of bonds was observed. Extensive hydrolysis occurred between aspartyl and proline residues (positions 28–29, 29–30, 199–200 and 312–313) and also between a pair of asparaginyl residues (127 and 128); the acid lability of aspartyl-proline bonds had been reported previously (Langley and Smith, 1971). More surprisingly, cleavage was observed at five tryptophan residues (48, 122, 205, 295 and 319) and at tyrosine 380. It is not clear whether the tryptophan residues were cleaved out of the chain or converted into uni-

dentifiable derivatives since, for example, Trp205 was not present at the N–terminus of peptide CN5 or in an identifiable form at the C–terminus of peptide CN4. The mechanism of cleavage at these points is obscure. It seems unlikely to be the result of acid hydrolysis (see below) and may represent a previously unreported side reaction of the cyanogen bromide procedure. It is interesting in this connection that tryptophanyl and tyrosyl bonds are susceptible to cleavage by bromine in addition to the more usual reagent N–bromosuccinimide (Ramachandran and Witkop, 1967). Hence in the present case cleavage may have occurred at particularly susceptible tryptophanyl and tyrosyl bonds due to bromine formation by decomposition of cyanogen bromide.

Incomplete cleavage at methionine and partial cleavage at other positions greatly limited the usefulness of the cyanogen bromide method in the solution of the structure of aspartate aminotransferase. The very complex mixture of large peptides proved difficult to separate and the extensive purification procedures resulted in very low yields of purified products. Some useful information was obtained, however, particularly in the case of peptide CN2 which provided an essential overlap in the N–terminal region of the molecule (Bossa *et al.*, 1973).

Cleavage with dilute acid was undertaken to exploit the apparently high lability of aspartyl-proline bonds under acid conditions. Fragmentation was observed at all the aspartyl-proline bonds and also at two asparaginyl-proline bonds. Cleavage was not observed at the bond between Asn–194 and Pro195 but since no peptide was isolated containing these two residues the occurrence of hydrolysis cannot be ruled out. It is apparent that although the conditions for digestion were designed to maximize hydrolysis of aspartyl-proline bonds, cleavage was not complete. For example, peptide A1a contains two intact aspartyl-proline bonds. On the other hand, many bonds were hydrolyzed other than those intended. Most of these involved either aspartyl or asparaginyl residues and were consistent with previous observations on the action of dilute acid on proteins (Schultz, 1967) although the conditions used here were milder than those generally employed (e.g. 0.03 M HCl, 105°, 4–24 hours). Other cleavage points were much more unexpected; for example hydrolysis of the following peptide bonds occurred: Arg113–Ile114, Arg159–Tyr160, Trp205–Lys206, Phe216–Leu217. It is not possible to state the degree of hydrolysis at these positions in the peptide chain due to the difficulties experienced in isolation of the fragments. The observed acid lability of these peptide bonds is unusual, even if it occurred to only a limited extent, and must reflect an influence on the stability of the bonds due to adjacent amino acid residues. Cleavage at the bond between Trp–205 and Lys–206 is of interest in connection with the results obtained in the digestion with cyanogen bromide. Peptide A6b contained the tryptophan unmodified at its C–terminus whereas this was not the case for peptides such as CN4. Hence it seems unlikely that cleavage at tryptophan during the cyanogen bromide reaction was due to partial acid hydrolysis.

In summary, it appears that although aspartyl–proline bonds are more labile to acid than other peptide bonds, the difference is not sufficiently great to constitute a method for specific chemical cleavage of proteins. Under conditions such that hydrolysis of aspartyl–proline bonds is extensive, a variety of other bonds are partially hydrolyzed producing a complex mixture of peptides with concomitant difficulty of purification of individual components.

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