The Disulphide Bridges of Bovine Chymotrypsinogen B

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The five cystine peptides of chymotrypsinogen B have been isolated as pairs of cysteic acid peptides by the diagonal electrophoretic technique of Brown & Hartley (1963, 1966). The sequences of these peptides have been shown to be very similar to those of chymotrypsinogen A, and indicate that both zymogens have the same pattern of disulphide bridges. The determination of the sequence of residues 14–21 in chymotrypsinogen B has shown that residue 18 differs in the chymotrypsinogens from the corresponding residue in trypsinogen. Eight differences between chymotrypsinogens A and B are found in sequences accounting for 72 of the 245 residues.

Bovine chymotrypsinogen B has many properties in common with chymotrypsinogen A. Both zymogens are found in equal amounts in bovine pancreatic juice (Keller, Cohen & Neurath, 1958), are very similar in size and shape (Guy, Gratecos, Rovey & Desnuelle, 1966; Smillie, Enenkel & Kay, 1966), have identical *N*- and *C*-terminal sequences (Kassell & Laskowski, 1962; Guy *et al.* 1966; Smillie & Hartley, 1965) and are activated to enzymes with only slightly dissimilar specificities towards polypeptides (Enenkel & Smillie, 1963; Kassell, Radicevic, Ansfield & Laskowski, 1965). They differ, however, in their rates of activation by trypsin (Krehbiel, Kassell & Laskowski, 1964; Guy *et al.* 1966), in their isoelectric points (Kubachi, Brown & Laskowski, 1949) and in their amino acid compositions (Kassell & Laskowski, 1961; Smillie *et al.* 1966; Guy *et al.* 1966).

By the diagonal electrophoretic technique of Brown & Hartley (1963, 1966) it is possible to isolate and identify the cystine sequences of the chymotrypsinogens. The peptides so isolated are of particular interest since they include sequences about those parts of the molecule believed to be involved in the active site of these enzymes: the 'active serine' and the histidines. Further, the cystine sequences of these proteins may be of particular importance in the determination of their tertiary structures. For these reasons we have applied the diagonal technique to chymotrypsinogen B as a first step in the elucidation of the primary structure of this protein. The results of this study have been briefly reported elsewhere (Smillie & Hartley, 1965) and the present paper gives the experimental evidence for these findings.

MATERIALS AND METHODS

Materials. These were as previously described (Brown & Hartley, 1966; Smillie & Hartley, 1966). Subtilisin, however, was the enzyme marketed as Nagarse by Biddle Sawyer Corp., New York, N.Y., U.S.A.

Diagonal electrophoretic peptide 'maps'. Diagonal electrophoretic peptide 'maps' of cysteic acid peptides from peptic digests of chymotrypsinogen B were prepared as described previously (Brown & Hartley, 1966; Smillie & Hartley, 1966). The digest was fractionated as a band on Whatman no. 3MM paper by high-voltage electrophoresis at pH 6.5. Marker strips cut from this sheet were oxidized with performic acid vapour, stitched to a larger sheet and submitted to electrophoresis at pH 6.5 at right angles to the original direction. Cysteic acid peptides derived from a single cystine peptide are revealed as parallel pairs of spots lying off the diagonal (e.g. Fig. 1) when such sheets are stained with cadmium–ninhydrin reagent (Heilmann, Barollier & Watzke, 1957).

Cysteic acid peptides from band 1A (Fig. 1). A band (1A) containing the cystine parents of peptides 1A1, 1A2 and 1A3 was cut from the first sheet, oxidized with performic acid vapour, stitched to another sheet of paper and submitted to electrophoresis at pH 6.5 for 2 hr. at 60 v/cm., perpendicular to the strip. Bands corresponding to peptides 1A1, 1A2 and 1A3 were revealed by staining marker strips with ninhydrin. These were each cut out and stitched again to another sheet of paper for electrophoresis at pH 3.5 (3 hr. at 60 v/cm.) to separate 1A2 from 1A3 and to purify 1A1 from 'diagonal' contaminants. The purified cysteic acid peptides were then eluted.

Cysteic acid peptides from band 1B (Fig. 1). The band containing the cystine precursors of peptides 1B1–1B7 was oxidized and submitted to electrophoresis at pH 6.5 for 1 hr. at 60 v/cm. Peptides 1B1 and 1B2 were not examined further, since their sequences had previously been determined (Smillie & Hartley, 1964, 1966). Peptides 1B3 and 1B5 were purified by electrophoresis at pH 6.5 for 3.5 hr. at

60 v/cm. Peptide 1B4 gave three acidic peptides 1B4A, 1B4B and 1B4C when fractionated by electrophoresis at pH 3.5 for 4 hr. at 60 v/cm. Peptides 1B6 and 1B7 were not recovered in adequate yields for further investigation.

Tryptic digestion of peptides eluted from band 1C (Fig. 1). The area corresponding to band 1C was eluted from several sheets of pH 6.5 electrophoretograms of the peptic digest. The peptides, corresponding to the yield from 200 mg. of chymotrypsinogen B, were digested in 5 ml. of 0.1 M-pyridine-acetate buffer, pH 6.5, with 2 mg. of trypsin at room temperature overnight. A pH 6.5/6.5 diagonal peptide 'map' of this digest was prepared in the usual way (Fig. 2).

Cysteic acid peptides from bands 2A and 2B (Fig. 2). Peptides 2A1, 2A2, 2A3 and 2A5 were prepared by oxidation of band 2A and electrophoresis at pH 6.5 in the usual way. Peptide 2A4 was found to be impure and gave, by electrophoresis at pH 3.5 for 90 min. at 60 v/cm., peptide 2A4A (neutral) and peptides 2A4B and 2A4C (both anionic).

Band 2B, after oxidation and electrophoresis at pH 6.5, gave peptides 2B1, 2B2, 2B3 and 2B4. Peptide 2B4 contains two cysteic acid residues as shown in Table 2. Digestion of this peptide with subtilisin (250 μ moles of peptide and 0.4 μ mole of subtilisin in 0.5 ml. of pyridine-acetate buffer, pH 6.5, for 16 hr. at 25°) gave two fragments, 2B4S1 and 2B4S2. These were separated by electrophoresis at pH 6.5 as a 10 cm. band on Whatman no. 1 paper for 1 hr. at 60 v/cm. Peptide 2B4S2 was anionic with a mobility consistent with the presence of two negative charges, and peptide 2B4S1 was neutral. The latter was purified by electrophoresis at pH 1.8 for 30 min. at 60 v/cm. The compositions are shown in Table 2 and the electrophoretic mobilities indicate that peptide 2B4S1 has a glutamine residue and 2B4S2 has an aspartic acid residue.

Subtilisin digestion of peptides eluted from band 2B. From these preliminary analyses it was clear that band 2B consisted of four half-cystine sequences, which in the native structure could have been bridged in any one of three ways. To resolve this ambiguity, it was necessary to split the unoxidized polypeptide chain between the half-cystine residues corresponding to peptide 2B4 and to separate the resulting cystine peptides. For this purpose subtilisin digestion of band 2B was carried out.

An amount of band 2B, corresponding to the yield from 16 μ moles of chymotrypsinogen B, was eluted from several sheets and freeze-dried. This was dissolved in 3.5 ml. of pyridine-acetate buffer, pH 6.5, 0.05 μ mole of subtilisin in 100 μ l. of water was added and the digest was incubated at 37° for 20 hr. A preliminary pH 6.5 diagonal experiment indicated that in addition to some acidic and basic cystine peptides there was a complex mixture of neutral or almost neutral cystine peptides migrating close to the origin in the first dimension. For this reason the remainder of the subtilisin digest was applied as a 20 cm.-wide band on Whatman no. 1 paper and subjected to electrophoresis at pH 6.5 for 90 min. (double the usual time) at 60 v/cm. After performic acid oxidation in the usual way, a 3 cm. strip was subjected to pH 6.5 electrophoresis in a direction perpendicular to the original electrophoresis for 45 min. at 60 v/cm. The resulting diagonal 'map' is shown in Fig. 3.

Cysteic acid peptides from bands 3A, 3B, 3C, 3D and 3E (Fig. 3). Each of the cysteic acid peptides indicated in Fig. 3 was prepared by oxidation of the appropriate band (3A, 3B, 3C, 3D or 3E), followed by electrophoresis at

pH 6.5. Further electrophoresis at pH 3.5 for 2 hr. at 60 v/cm. caused peptide 3D3 to separate into three anionic peptides 3D3A, 3D3B and 3D3C. The neutral peptide 3E1 was repurified by electrophoresis at pH 1.8 for 30 min. at 60 v/cm.

Analysis and sequence-determination methods. These were as previously described (Smillie & Hartley, 1966).

RESULTS

A comparison of pH 6.5/6.5 diagonal peptide 'maps' of peptic digests of chymotrypsinogens B and A is shown in Fig. 1, where the nomenclature of cysteic acid peptides is the same as that used by Brown & Hartley (1966). Certain resemblances are immediately apparent. Band 1A is less acidic than the corresponding band in chymotrypsinogen A but shows the same pattern of cysteic acid peptides. Thus 1A1 is a yellow peptide, 1A2 is red and 1A3 developed slowly to a red colour. Moreover, peptides 1B1 and 1B2 are in positions identical with the same peptides of the chymotrypsinogen A 'map', are both Pauly-positive and are vertically in line with one another but slightly displaced from the other peptides of band 1B. The composition and sequence of these two peptides, which form the 'histidine loop' of chymotrypsinogen A, has previously been reported (Smillie & Hartley, 1966). The other peptides of bands 1B and 1C bear no immediately obvious relationship to the bands of the chymotrypsinogen A digest.

Peptides from band 1A. Our evidence for the sequence* of peptides 1A1, 1A2 and 1A3 is summarized in Table 1. The release of essentially molar amounts of glutamine and lesser amounts of isoleucine by carboxypeptidase A confirmed the C-terminal sequence of peptide 1A1. Since the original cystine peptides of band 1A were acidic at pH 6.5, the C-terminal residue of peptides 1A2 and 1A3 must be aspartic acid and not asparagine. This was confirmed by the isolation of an acidic peptide containing only alanine and aspartic acid from a subtilisin digest of peptide 1A2. It could be concluded therefore that the cysteic acid peptides of band 1A were derived from a cystine peptide:

which is homologous with the CyS-1-CyS-122 disulphide bridge of chymotrypsinogen A.

Peptides from band 1B. The histidine peptides 1B1 and 1B2 were not examined further since they had been previously isolated and their sequences

* Abbreviations (in amino acid sequences): CySO₃H, cysteic acid; MetSO₂, methionine sulphone; Asx, aspartic acid or asparagine; Glx, glutamic acid or glutamine.

Fig. 1. Diagonal peptide 'map' of a peptic digest of chymotrypsinogens A and B. Electrophoresis was at pH 6.5 in both dimensions. After electrophoresis in the first dimension, a marker strip was removed from the sheet of paper, oxidized with performic acid vapour and sewn to another sheet, which was submitted to electrophoresis at right angles to the original direction. The origin is the vertical line in the middle of the sewn strip. Cysteic acid peptides are cross-hatched.

Table 1. Amino acid composition and sequence of the peptides isolated from the diagonal 'map' (Fig. 1) of a peptic digest of chymotrypsinogen B

The symbols $\longrightarrow \longrightarrow \dots$ indicate *N*-terminal analyses by the 'dansyl'-Edman procedure; $\dots \longleftarrow$ represents *C*-terminal analysis by carboxypeptidase. Compositions are expressed as uncorrected ratios of amino acids after 16 hr. hydrolysis *in vacuo* at 105°. In those cases (e.g. peptide 1A2) where the peptide contained more than one residue of a particular amino acid (e.g. alanine) the figure quoted was obtained by dividing the total amount of that amino acid by the number of residues.

Peptide	
1A1	CySO ₃ H-Gly-Val-Pro-Ala-Ile-Gln $\xrightarrow{0.95} \xrightarrow{1.06} \xrightarrow{0.95} \xrightarrow{1.17} \xrightarrow{1.11} \xrightarrow{0.89} \xrightarrow{1.06}$
1A2	Ala-Val-(CySO ₃ H,Leu,Pro,Ser,Ala,Asp) $\xrightarrow{0.85} \xrightarrow{0.89} \xrightarrow{1.30} \xrightarrow{1.00} \xrightarrow{1.08} \xrightarrow{0.85} \xrightarrow{0.95}$
1A3	Val-CySO ₃ H-Leu-Pro-Ser-Ala-Asp $\xrightarrow{0.90} \xrightarrow{0.87} \xrightarrow{0.90} \xrightarrow{1.30} \xrightarrow{0.93} \xrightarrow{1.11} \xrightarrow{1.03}$
1B1	His-Phe-CySO ₃ H-Gly-Gly-Ser-Leu
1B2	Ala-Ala-His-CySO ₃ H-Gly-Val-Thr-Thr
1B3	(Ala,Val,CySO ₃ H,Leu,Pro,Ser) $\xrightarrow{1.28} \xrightarrow{0.77} \xrightarrow{1.16} \xrightarrow{0.80} \xrightarrow{0.87} \xrightarrow{1.13}$
1B4A	(Ser,CySO ₃ H,MetSO ₂ ,Gly,Asp,Ser,Gly,Gly,Pro,Leu,Val,CySO ₃ H,Glu,Lys,Asp,Gly,Ala)
1B4B	(CySO ₃ H,Gly,Val,Pro,Ala,Ile,Glu)
1B4C	Leu-CySO ₃ H-Ala-Thr-Thr-Gly $\xrightarrow{0.89} \xrightarrow{1.00} \xrightarrow{1.11} \xrightarrow{1.00} \xrightarrow{1.00} \xrightarrow{1.11}$
1B5	(Ala,Val,CySO ₃ H,Leu) $\xrightarrow{0.89} \xrightarrow{0.94} \xrightarrow{1.09} \xrightarrow{1.06}$

determined (Smillie & Hartley, 1964, 1966). Peptides 1B3, 1B5 and 1B4C were subjected to quantitative amino acid analysis and peptides 1B4A and 1B4B were examined qualitatively for amino acid content by two-dimensional paper electrophoresis and chromatography, with the results shown in Table 1. From these analyses it was clear that peptide 1B4B was identical with the previously isolated peptide 1A1, the sequence of which had been determined, and that peptides 1B3 and 1B5 were smaller varieties of peptides 1A2 and 1A3. Qualitative amino acid analysis of peptide 1B4A indicated that it corresponded to residues 190–206 of the sequence of chymotrypsinogen A except that one lysine residue had been replaced by a glutamic acid or glutamine residue. Only peptide 1B4C was recovered in adequate amounts for the determination of its sequence by the 'dansyl'-Edman procedure, with the results shown in Table 1.

It was clear from these preliminary analyses that band 1B was a complex mixture of several neutral cystine peptides involving half-cystines corresponding to residues 1, 42, 58, 122, 191, 201 and probably 136 and 220 of the chymotrypsinogen A sequence (Hartley, 1964; Hartley & Kauffman, 1966). No further analysis of these peptides was attempted since they were difficult to purify and to recover in adequate yield for full characterization.

Tryptic peptides from band 1C. Preliminary analysis of the cysteic acid peptides from band 1C indicated that these were a complex mixture consisting of probably six different half-cystine sequences. For this reason the peptides from band 1C were eluted and digested with trypsin. Fig. 2 shows the diagonal 'map' of this tryptic digest, which contains two bands of cysteic acid peptides. These peptides (2A1–2A5 and 2B1–2B4) were isolated as described above and Table 2 summarizes our studies of their composition and sequence.

Band 2A clearly represents a group of cysteic acid peptides arising from the sequence:

Asn-Thr-Asp-CyS-Arg

Met-Ile-CyS-Ala-Gly-Ala-(Ser,Gly,Val,Ser)

The cystine precursors were neutral at pH 6.5 and hence must contain one asparagine residue and one aspartic acid residue to balance the charge of the arginine. The aspartic acid residue must be next to the half-cystine since peptides 2A4A and 2A5 were both anionic at pH 6.5. Moreover, the electrophoretic mobility of peptide 2A3 at pH 6.5 was slightly less than that of peptide 2A4A, showing that the extra *N*-terminal residue must be asparagine.

Band 2B is slightly cationic and contains four cysteic acid sequences. Peptide 2B1 appears to

Fig. 2. Diagonal peptide 'map' of a tryptic digest of cystine peptides eluted from band 1C of chymotrypsinogen B. Electrophoresis was at pH 6.5 in both dimensions. After electrophoresis in the first dimension, a marker strip was removed from the sheet of paper, oxidized with performic acid vapour and sewn to another sheet, which was submitted to electrophoresis at right angles to the original direction. The origin is the vertical line in the middle of the sewn strip. Cysteic acid peptides are cross-hatched.

contain an oxidized tryptophan residue because of its strong fluorescence, and its slightly anionic mobility is probably due to the low *pK* of the *N*-terminal cysteic acid. Peptide 2B2 contains a different cysteic acid sequence characterized by a high content of serine and threonine. Qualitative analysis showed peptide 2B3 to be similar to peptide 2B4, with a higher valine and serine content, and 'dansyl'-Edman analysis gave the *N*-terminal sequence Val-Ser-Ser. We believe that peptide 2B3 corresponds to peptide 2B4 with an extra Val-Ser at the *N*-terminus.

Subtilisin peptides from band 2B. Fig. 3 shows the diagonal 'map' of the subtilisin digest of the cystine peptides eluted from the position of band 2B (Fig. 2). Each of the peptides indicated was isolated as described above, and Table 3 summarizes our studies of their composition and sequence.

Peptides 3D2, 3D3A, 3D4, 3E3, 3E4, 3E5 and 3E6 are clearly smaller varieties of peptide 1B4C (Table 1). It is also evident that peptides 3A2, 3B2, 3C1, 3D1, 3D3C, 3E1 and 3E2 are smaller varieties of peptide 2B4 (Table 2) arising from multiple subtilisin cleavages of peptide bonds between the two cysteic acid residues of the latter peptide. It is thus possible to write the following structure for the two remaining disulphide bridges of chymotrypsinogen B:

Table 2. Amino acid composition and sequence of the peptides isolated from the diagonal 'map' (Fig. 2) of the tryptic digest of the cystine peptides eluted from band 1C (Fig. 1)

The symbols and compositions are defined in Table 1.	
Peptide	
2A1	Ile - CySO ₃ H-Ala - Gly - Ala - (Ser,GlyVal,Ser) $\xrightarrow{0.58} \xrightarrow{0.66} \xrightarrow{0.96} \xrightarrow{1.01} \xrightarrow{0.96} \xrightarrow{1.05} \xrightarrow{1.01} \xrightarrow{0.92} \xrightarrow{1.05}$
2A2	MetSO ₂ -Ile - CySO ₃ H-Ala - Gly - (Ala,Ser,Gly,Val,Ser) $\xrightarrow{0.88} \xrightarrow{0.81} \xrightarrow{0.88} \xrightarrow{1.00} \xrightarrow{1.06} \xrightarrow{1.00} \xrightarrow{0.97} \xrightarrow{1.06} \xrightarrow{1.19} \xrightarrow{0.97}$
2A3	Asn-Thr-Asp-CySO ₃ H-Arg $\xrightarrow{0.97} \xrightarrow{0.97} \xrightarrow{0.97} \xrightarrow{1.10} \xrightarrow{0.97}$
2A4A	Thr-Asp-CySO ₃ H-Arg $\xrightarrow{1.08} \xrightarrow{1.08} \xrightarrow{1.00} \xrightarrow{0.85}$
2A4B	MetSO ₂ -(Ile,CySO ₃ H,Ala,Gly,Ala,Ser,Gly) $\xrightarrow{0.64} \xrightarrow{0.56} \xrightarrow{0.96} \xrightarrow{0.92} \xrightarrow{0.96} \xrightarrow{0.92} \xrightarrow{1.36} \xrightarrow{0.96}$
2A4C	Ile-(CySO ₃ H,Ala,Gly,Ala,Ser,Gly) $\xrightarrow{0.90} \xrightarrow{1.08} \xrightarrow{0.95} \xrightarrow{1.05} \xrightarrow{0.95} \xrightarrow{1.02} \xrightarrow{1.06}$
2A5	Asp-CySO ₃ H-Arg $\xrightarrow{1.00} \xrightarrow{1.15} \xrightarrow{0.85}$
2B1	CySO ₃ H-(Ala,Thr,Thr,Gly,Trp,Gly,Lys) $\xrightarrow{0.77} \xrightarrow{1.18} \xrightarrow{1.09} \xrightarrow{1.09} \xrightarrow{0.89} \xrightarrow{+} \xrightarrow{0.89} \xrightarrow{1.18}$
2B2	Gly-(Ser,Ser,Thr,CySO ₃ H,Ser,Thr) $\xrightarrow{1.30} \xrightarrow{0.84} \xrightarrow{0.84} \xrightarrow{1.14} \xrightarrow{0.89} \xrightarrow{0.84} \xrightarrow{1.13}$
2B3	Val-Ser-Ser-(CySO ₃ H,MetSO ₂ ,Gly,Asp,Ser,Gly,Gly,Pro,Leu,Val,CySO ₃ H,Gln,Lys)
2B4	Ser-CySO ₃ H-MetSO ₂ -Gly-Asp-(Ser,Gly,Gly,Pro,Leu,Val,CySO ₃ H,Gln,Lys) $\xrightarrow{0.96} \xrightarrow{0.87} \xrightarrow{1.01} \xrightarrow{1.04} \xrightarrow{1.06} \xrightarrow{0.97} \xrightarrow{1.04} \xrightarrow{1.04} \xrightarrow{1.17} \xrightarrow{0.98} \xrightarrow{0.90} \xrightarrow{0.87} \xrightarrow{1.05} \xrightarrow{1.02}$
2B4S1	Val-(CySO ₃ H,Gln,Lys) $\xrightarrow{1.02} \xrightarrow{0.96} \xrightarrow{0.98} \xrightarrow{1.04}$
2B4S2	Ser-(CySO ₃ H,MetSO ₂ ,Gly,Asp,Ser,Gly,Gly,Pro,Leu) $\xrightarrow{0.88} \xrightarrow{0.95} \xrightarrow{1.04} \xrightarrow{0.99} \xrightarrow{0.92} \xrightarrow{0.88} \xrightarrow{0.99} \xrightarrow{0.99} \xrightarrow{1.06} \xrightarrow{1.05}$

Peptide G. In addition to the disulphide peptides isolated in this work a further peptide (peptide G, Fig. 1) was isolated and characterized. This peptide, present on the pH 6.5 diagonal of peptic digests of chymotrypsinogens A and B, was not observed when similar digests of the active enzymes were performed. It thus appeared likely that this peptide contained that portion of the structure

cleaved by trypsin during the activation process, namely the arginine-15-isoleucine-16 bond of the chymotrypsinogen A sequence.

Bands containing peptide G were cut out from several sheets and repurified by electrophoresis at pH 1.8 for 40 min. at 60 v/cm. After elution, the peptide was subjected to amino acid and 'dansyl'-Edman analysis as indicated in Table 4. From these

Fig. 3. Diagonal peptide 'map' of a subtilisin digest of cystine peptides eluted from band 2B of chymotrypsinogen B. After electrophoresis in the first dimension, a marker strip was removed from the sheet of paper, oxidized with performic acid vapour and sewn to another sheet, which was submitted to electrophoresis at right angles to the original direction. The origin is the vertical line in the middle of the sewn strip. Electrophoretic conditions were as described in the text. The major cysteic acid peptides are cross-hatched.

results it is possible to write the sequence for peptide G shown in Table 4, corresponding to residues 14-21 in the sequence of chymotrypsinogen A.

DISCUSSION

The homologies of sequence about the five disulphide bridges and residues 14-21 of chymotrypsinogens A and B are summarized in Table 5. As expected, homologous sequences are similarly disulphide-bridged in both proteins. Although many of the sequences are identical in the two proteins, sufficient differences are seen to establish the case that chymotrypsins A and B are 'iso-enzymes' derived from homologous but different genes in the same animal. Eight differences of sequence in the 72 residues shown in Table 5 would extrapolate to a total of 27 differences in the whole

Table 4. *Amino acid composition (70 hr. hydrolysate) and sequence of the peptide G (Fig. 1) isolated from the diagonal 'map' of a peptic digest of chymotrypsinogen B*

The symbols and compositions are defined in Table 1.

Ala - Arg - Ile - Val - Asx - Gly - (Glx, Asx)
 0.99 0.95 0.93 0.97 1.00 1.05 1.07 1.01

Table 3. *Amino acid composition and sequence of the peptides isolated from the diagonal map (Fig. 3) of a subtilisin digest of the cystine peptides eluted from band 2B (Fig. 2)*

The symbols and compositions are defined in Table 1.

Peptide		Peptide	
3A1	CySO ₃ H-Ser - Thr 1.10 1.00 0.92	3D3B	CySO ₃ H-(Ser,Thr) 1.01 1.01 0.99
3A2	Ser - CySO ₃ H-MetSO ₂ -Gly - Asp-Ser 0.98 0.98 0.95 1.13 1.03 0.97	3D3C	Val - CySO ₃ H 0.96 1.04
3B1	CySO ₃ H-(Ser,Thr) 1.17 0.88 0.92	3D4	CySO ₃ H-Ala 1.02 0.98
3B2	CySO ₃ H-MetSO ₂ 1.11 0.89	3E1	Val - CySO ₃ H-Gln - Lys 0.98 0.96 1.02 1.04
3C1	Ser - (CySO ₃ H, MetSO ₂) 1.00 1.10 0.90	3E2	CySO ₃ H-(Gln, Lys) 1.08 0.96 0.96
3C2	CySO ₃ H-(Ser,Thr) 1.18 0.86 0.96	3E3	Leu - CySO ₃ H-Ala - Thr 0.96 0.88 1.13 1.04
3D1	Val - (CySO ₃ H, Gln) 0.88 1.32 0.84	3E4	CySO ₃ H-(Ala, Thr, Thr) 1.08 1.10 0.91 0.91
3D2	CySO ₃ H-(Ala, Thr, Thr) 1.29 0.87 0.91 0.91	3E5	CySO ₃ H-(Ala, Thr) 1.05 0.97 0.98
3D3A	CySO ₃ H-(Ala, Thr) 0.96 0.96 1.09	3E6	CySO ₃ H-Ala 1.02 0.98

Table 5. *Cystine bridges in bovine chymotrypsinogens*

Residues in *italics* show differences in sequence between chymotrypsinogen B (ChB) and chymotrypsinogen A (ChA). Residues of chymotrypsinogen A are numbered as in Brown & Hartley (1966).

ChB
ChA

Cys-Gly-Val-Pro-Ala-Ile-Gln
Cys-Gly-Val-Pro-Ala-Ile-Gln
1 2 3 4 5 6 7

Ala-Arg-Ile-Val-Asx-Gly-(Glx,Asx)
Ser-Arg-Ile-Val-Asn-Gly-Glu-Glu
14 15 16 17 18 19 20 21

ChB
ChA

His-Phe-Cys-Gly-Gly-Ser-Leu
His-Phe-Cys-Gly-Gly-Ser-Leu
40 41 42 43 44 45 46

Ala-Ala-His-Cys-Gly-Val-Thr-Thr
Ala-Ala-His-Cys-Gly-Val-Thr-Thr
55 56 57 58 59 60 61 62

ChB
ChA

Ala-Val-Cys-Leu-Pro-Ser-Ala-Asp
Ala-Val-Cys-Leu-Pro-Ser-Ala-Ser-Asp-Asp
120 121 122 123 124 125 126 127 128 129

Leu-Cys-Ala-Thr-Thr-Gly
Thr-Cys-Val-Thr-Thr-Gly
135 136 137 138 139 140

ChB
ChA

Asn-Thr-Asp-Cys-Arg
Asn-Thr-Asn-Cys-Lys
165 166 167 168 169

Met-Ile-Cys-Ala-Gly-Ala
Met-Ile-Cys-Ala-Gly-Ala
180 181 182 183 184 185

ChB
ChA

Ser-Cys-Met-Gly-Asp-Ser-(Gly,Gly,Pro,Leu)-Val-Cys-Gln-Lys
Ser-Cys-Met-Gly-Asp-Ser-Gly-Gly-Pro-Leu-Val-Cys-Lys-Lys
190 191 192 193 194 195 196 197 198 199 200 201 202 203

ChB
ChA

Cys-Ser-Thr
Cys-Ser-Thr
220 221 222

chain of 245 residues. However, the final total of differences is likely to be considerably higher than this, since the cysteine acid peptides include both the histidine sequences and the active serine sequence that form part of the active centre. These structures are likely to be more conservative than the rest of the chain.

Perhaps the main use of a comparison of the

sequences of the two zymogens will be in delineating lengths of sequence that are not essential for their similar enzymic activities. In this sense, chymotrypsin B can be considered to carry built-in chemical modifications of chymotrypsin A, and residues that differ in the two proteins can be excluded from a critical role in the activity or in the maintenance of a common tertiary structure. That

the two proteins have very similar, though not identical, tertiary structures is indicated by recent studies of the optical-rotatory-dispersion properties of the two zymogens (Smillie *et al.* 1966). In the light of these considerations, it is not surprising that the structures near the disulphide bridges, which must in part determine their secondary and tertiary structures, have very similar sequences in the two proteins. However, those residues that are radically different in the two structures could in most cases be considered to occur on the 'outside' of the molecule, since such changes could be most easily tolerated here. In this way we think that such comparisons of isoenzyme sequences may help in mapping groups that lie at the surface of the protein molecule. From a comparison of the structures presented in Table 5, it is apparent that there is only one disulphide bridge on both sides of which there are significant differences in amino acid sequence. Thus at positions 135 and 137 there are replacements of threonine by leucine and of valine by alanine respectively, and at position 202 lysine is replaced by glutamine. The hypothesis that this disulphide bond lies at or near the surface of the molecule is supported by the availability of all of the lysine residues of chymotrypsinogen A to reaction with *O*-methylisourea (Chervenka & Wilcox, 1956). Thus both residue 202 and residue 203 are presumably at the surface of the chymotrypsinogen A and B molecules.

The elucidation of the sequence of peptide G (residues 14–21) has shown that, in chymotrypsinogen B, the new *N*-terminal sequence Ile-Val-Asx-Gly formed during the tryptic activation of the zymogen by cleavage of the 15–16 peptide bond is identical with the trypsin sequence at residues 16 and 17 but not at residue 18. This discovery has resulted in a recent reinvestigation of this sequence in chymotrypsin A, which has shown it to be identical with the sequence in the B enzyme (Hartley & Kauffman, 1966) and therefore different from trypsin at position 18.

Differences in the primary structure of the two zymogens A and B, in the immediate region of the site of tryptic activation of the two proteins, are of considerable interest because of certain differences in the kinetics and events of this activation. Thus although the initial chemical event involving the tryptic cleavage of the arginine-isoleucine bond at residues 15 and 16 is the same in both zymogens, the rate of this cleavage in chymotrypsinogen B is some 15-fold faster than in chymotrypsinogen A (Guy *et al.* 1966; Krehbiel *et al.* 1964). Further, the subsequent chymotryptic autolysis of the leucine-13-serine-14 peptide bond does not occur in chymotrypsin B (Guy *et al.* 1966; Krehbiel *et al.* 1964). From the present work and from other studies (Furka, Smillie, Stevenson & Parkes, 1966),

it has become evident that the replacement of serine-14 in chymotrypsinogen A by alanine in chymotrypsinogen B is the only significant change in the A and B proteins in residues 1–34. How this replacement may affect the rate of tryptic activation and the resistance of the 13–14 peptide bond to autolysis in chymotrypsin B is conjectural. However, if it is assumed that this region of the polypeptide chain is sufficiently flexible to meet the steric requirements of the active site of trypsin, it may be postulated that such flexibility is reduced in chymotrypsinogen A by the presence of serine at position 14. Thus serine-14 in chymotrypsinogen A may be hydrogen-bonded to some other residue lying on or near the surface of the protein molecule. Such bonds involving the side chains of serine residues have been observed in the X-ray-diffraction studies of myoglobin (Kendrew, 1962).

Alternatively, if it is assumed that this region of the chain is a rigid structure locked into a configuration closely fitting the requirements of the active site of trypsin, then the replacement of serine-14 by alanine-14 must produce a structure meeting these requirements more exactly. Hopefully, elucidation of the tertiary structures of chymotrypsinogen and chymotrypsin will provide an answer to these questions.

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