Analysis of the heterogeneity of human collagens by two-dimensional polyacrylamide-gel electrophoresis

William G. COLE* and Danny CHAN

Department of Paediatrics, University of Melbourne, Royal Children's Hospital, Melbourne, Vic. 3052, Australia

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The heterogeneity of the CNBr-cleavage peptides of human types I, II, III and V collagens were studied by using two-dimensional electrophoresis combining non-equilibrium pH-gradient-gel electrophoresis and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Specific 'maps' were produced by the peptides obtained from the chains of each type of collagen, and most peptides had at least three charged forms of the same molecular weight. Specific 'maps' were also produced by the peptides of types I, III and V collagens from insoluble dermis and the peptides of types I and V collagens from decalcified bone. The $\alpha 1(I)$ CB7 and $\alpha 1(I)$ CB8 and the $\alpha 2$ CB4 peptides obtained from the type I collagens of these tissues contained the same number of charged components, but there was a relative increase in the more basic components in bone. Some aspects of the involvement of the $\alpha 1(I)$ CB6 and the $\alpha 1(III)$ CB9 peptides in cross-linkages were also studied. The recovery of the $\alpha 1(I)$ CB6 peptide from bone and dermis was decreased and the $\alpha 1(III)$ CB9 peptide was not detected in dermis. Additional peptides, which were probably cross-linked peptides involving the $\alpha 1(I)$ CB6 peptide, were also observed.

The collagens are a heterogeneous group of proteins consisting of at least nine genetically distinct chains that undergo extensive post-translational modifications (Bornstein & Sage, 1980). With a view to facilitating the study of these aspects of collagen structure in human connective-tissue diseases, we have analysed the heterogeneity of human collagens by the technique of high-resolution two-dimensional electrophoresis developed by O'Farrell *et al.* (1977). This procedure, which combines non-equilibrium pH-gradient-gel electrophoresis and SDS/polyacrylamide-gel electrophoresis, resolves proteins with isoelectric points across the entire pH range (O'Farrell *et al.*, 1977).

CNBr-cleavage peptides of collagen were selected for study, as it was considered more likely that charge heterogeneities would be detected with the peptides rather than the parent α -chains. We describe the specific CNBr-cleavage peptide 'maps' obtained from purified $\alpha 1(I)$ -, $\alpha 1(II)$ -, $\alpha 1(III)$ -, $\alpha 2$ -, αA - and αB -chains by this technique. We show that most peptides have at least three charged forms and that differences exist in the charge heterogeneity of

the peptides of type I collagen obtained from dermis and bone. We also show that the heterogeneity of the collagens, as well as some aspects of peptide involvement in cross-linkages, can be studied in small samples of dermis and bone by using this method of two-dimensional electrophoresis.

Experimental

Materials

Ampholines were supplied by LKB Instruments, Rockville, MD, U.S.A. Sources of other materials used have been previously described (Cole & Bean, 1979).

Preparation of human collagen CNBr-cleavage peptides

Types I, III and V collagens were prepared from dermis collected at autopsy from infants who had died from diseases that did not affect the connective tissues. Collection of these specimens was approved by the Ethics Committee of this hospital. Types I and III collagens were purified and separated by previously described methods (Miller, 1971; van der Rest *et al.*, 1977). Type V collagen was purified and separated into αA - and αB -chains as described by

Abbreviation used: SDS, sodium dodecyl sulphate.

* To whom reprint requests should be addressed.

Bentz *et al.* (1978) and von der Mark & von der Mark (1979). Type II collagen was prepared from human costal cartilage by previously described methods (Miller, 1971; Burgeson & Hollister, 1979).

CNBr cleavage of these collagens was performed by the method described by Scott & Veis (1976). Samples (2–5 mg) of insoluble dermis and decalcified bone were also prepared and cleaved with CNBr by previously described procedures (Cole & Bean, 1979). The individual CNBr-cleavage peptides of the $\alpha 1(I)$ -, $\alpha 1(II)$ -, $\alpha 1(III)$ - and $\alpha 2$ -chains were also prepared by the methods described by Miller (1972) and Lichtenstein *et al.* (1975). Peptides $\alpha 1(I)$ CB7 and $\alpha 1(I)$ CB8 were rechromatographed on CM-cellulose as described by Butler *et al.* (1967).

Non-equilibrium pH-gradient first-dimension electrophoresis

The buffers, solutions and procedures used were as described by O'Farrell *et al.* (1977). The gel mixture contained 1.8% (v/v) of pH 3.5–10 Ampholines and 0.2% (v/v) of pH 5–8 Ampholines. The CNBr-cleavage peptides were dissolved (10 mg/ml) in sample buffer, and 5 μ l of 20 mM-NaOH was added to each 50 μ l of sample to convert homoserine lactone into homoserine (Finlayson & Chrambach, 1971); 10–20 μ l of sample was loaded on to each gel. Electrophoresis was conducted at 500 V for 3 h at room temperature, and at the completion of the run the gels were removed from the tubes and placed in 5 ml of an SDS sample buffer for 30 min at room temperature (O'Farrell *et al.*, 1977). Gels were either stored in this solution at -20°C until analysed or used immediately.

SDS/polyacrylamide-slab-gel second-dimension electrophoresis

The vertical slab-gel apparatus described by O'Farrell (1975) was used. Polyacrylamide-gel slabs (150 mm length, 180 mm wide and 0.8 mm thick) were cast with an exponential gradient of acrylamide from 11 to 16% (w/v), and a 50 mm stacking gel of 4.5% (w/v) acrylamide was applied to the top of the resolving gel. Electrophoresis was performed at 7 mA/gel for 30 min and at 25 mA/gel until the dye front had reached the end of the gel. Each run was usually complete after 5 h at 4°C . The slab gels were stained with Coomassie Brilliant Blue and destained as previously described (Cole & Bean, 1979).

Results

Conditions of electrophoresis

All of the peptides of type I collagen normally

Fig. 1. Two-dimensional electrophoresis of CNBr-cleavage peptides of the $\beta 12$ -chain

A sample (100 μ g) containing the CNBr-cleavage peptides of the $\beta 12$ -chain was resolved by non-equilibrium pH-gradient gel electrophoresis and SDS/polyacrylamide-gel electrophoresis as described in the text. The $\alpha 1(I)$ - and $\alpha 2$ -chain peptides are indicated. The peptides marked (1) probably represent incompletely cleaved sequences of the $\alpha 1(I)$ -chain.

seen on SDS/polyacrylamide-gel electrophoresis were observed in the second-dimension gel (Fig. 1). As all the peptides were located within the linear part of the pH gradient (pH 4.2–9.2), these conditions of electrophoresis were used throughout this study. The 'maps' obtained from multiple runs of the same as well as different preparations of peptides of dermal type I collagen were highly reproducible and in most instances identical. Differences were not observed with different batches of LKB Ampholines of the same pH range. In addition, differences were not observed in the 'maps' obtained with equilibrated gels run immediately or stored for up to 3 months at -20°C .

When isoelectric focusing was used instead of non-equilibrium pH-gradient gel electrophoresis, only a portion of the peptide 'map' could be studied at one time, owing to changes in the pH gradient with prolonged electrophoresis (O'Farrell, 1975). After electrophoresis for 7300 V·h, which was within the range of 5000–10000 V·h recommended by O'Farrell (1975) for isoelectric focusing, the pH gradient with pH 3.5–10 Ampholines was linear only from pH 4.1 to pH 6.2, and with pH 7–11 Ampholines the pH gradient was linear from pH 7.3 to pH 9.1.

CNBr-cleavage peptides from purified collagens

The $\alpha 1(I)$ CB3, CB6, CB7 and CB8 peptides from the $\alpha 1(I)$ -chain of type I collagen were well separated (Fig. 1), and their identities were confirmed by using purified peptides. These peptides represent approx. 86% of the chain, whereas the $\alpha 1(I)$ CB0.1, CB2, CB4 and CB5 peptides, which are located in the *N*-terminal end of the chain, have molecular weights less than 5000 and could not be studied with this gel system (Scott & Veis, 1976). The peptides marked (1) in Fig. 1 were probably incompletely cleaved sequences such as the CB4–CB5–CB8–CB3 and the CB5–CB8–CB3 peptides described by Scott & Veis (1976).

The pH gradient is indicated in Fig. 1, but these values do not represent the isoelectric points of the peptides, as they did not reach equilibrium in this system. The peptides were distributed along the pH gradient in the order CB3, CB6, CB7 and CB8, which corresponds to their order of elution during CM-cellulose chromatography (Butler *et al.*, 1967).

Each peptide had at least three charged forms of the same molecular weight. This was a constant finding with different preparations of $\alpha 1(I)$ peptides. Reference to the charged standards, prepared as described by Anderson & Hickman (1979), showed that each charged form differed by three or four charges from its neighbours. The multiple forms of

peptide $\alpha 1(I)$ CB7 were studied in more detail. Purified peptide $\alpha 1(I)$ CB7 was separated into three components, designated peptides $\alpha 1$ CB7', 7 and 7'', by rechromatography on CM-cellulose in sodium acetate buffers without urea (results not shown). Peptide $\alpha 1$ CB7' contained the acidic components and peptide $\alpha 1$ CB7'' the basic components on two-dimensional electrophoresis (Fig. 2). Similar findings were observed with the $\alpha 1(I)$ CB8 peptide (results not shown).

The larger peptides obtained from the $\alpha 2$ -chain of a $\beta 12$ dimeric mixture of $\alpha 1(I)$ - and $\alpha 2$ -chains are shown in Fig. 1. The identities of the $\alpha 2$ CB3.5 and CB4 peptides were confirmed by using purified peptides. The patterns obtained were reproducible with different preparations of $\beta 12$ - and purified $\alpha 2$ -chains, and the relative positions of the $\alpha 2$ and $\alpha 1(I)$ peptides were also constant. The $\alpha 2$ CB3.5 and the $\alpha 1(I)$ CB7 peptides migrated to a similar position in the pH gradient, although peptide $\alpha 2$ CB3.5 was eluted after peptide $\alpha 1(I)$ CB7 during CM-cellulose chromatography. This discrepancy may be due to greater retardation of the $\alpha 2$ CB3.5 peptide in the first-dimensional gel because of its higher molecular weight (Scott & Veis, 1976).

The $\alpha 2$ CB3.5 and CB4 peptides account for nearly 95% of the $\alpha 2$ -chain (Scott & Veis, 1976).

Fig. 2. Two-dimensional electrophoresis of the rechromatographed $\alpha 1(I)$ CB7 peptide

The $\alpha 1(I)$ CB7 peptide was rechromatographed as described in the text. The components designated $\alpha 1$ CB7', $\alpha 1$ CB7 and $\alpha 1$ CB7'' were obtained. Each component, as well as a mixture of the components, was resolved by non-equilibrium pH-gradient gel electrophoresis and SDS/polyacrylamide-gel electrophoresis. Only the $\alpha 1(I)$ CB7 region of each gel is shown.

Fig. 3. Two-dimensional electrophoresis of CNBr-cleavage peptides of the $\alpha 1(III)$ -chain

A sample (100 μ g) of $\alpha 1(III)$ -chain CNBr-cleavage peptides was resolved by non-equilibrium pH-gradient gel electrophoresis and SDS/polyacrylamide-gel electrophoresis as described in the text. The positions of the faint CB4 peptides are marked with circles.

The low-molecular-weight peptides CB0, CB1 and CB2 were not detected in the system that we used. Both of the major peptides of the $\alpha 2$ -chain showed charge heterogeneity. Peptide $\alpha 2$ CB3.5 contained at least six components, whereas peptide $\alpha 2$ CB4 had two major components.

The $\alpha 1$ (III) peptides also produced a distinctive 'map' (Fig. 3). The positions of the larger peptides (CB4, CB5, CB8 and CB9) were confirmed by using purified peptides. These peptides constituted approx. 71% of the $\alpha 1$ (III)-chain (Chung *et al.*, 1974), whereas the smaller peptides (CB1, CB2, CB3, CB6, CB7 and CB10) were not detected with this gel system (Seyer *et al.*, 1980). The material with molecular weights greater than those of the CB5 and CB9 peptides probably represented incompletely cleaved peptide sequences.

The $\alpha 1$ (III) CB9 peptide, which is disulphide-linked in the type III collagen molecule, ran in the reduced form, because of inclusion of β -mercaptoethanol in the sample buffer. The CB4 peptide migrated to a more basic part of the pH gradient than expected from the CM-cellulose-chromatography elution profile of these peptides. In addition, this peptide stained a pink rather than a blue colour with the Coomassie Blue stain. As with the other interstitial collagens studied, these peptides showed multiple charged forms, and the patterns were constant for different preparations of peptides of type III collagen.

The peptide 'map' obtained from the $\alpha 1$ (II)-chain of type II collagen was distinct from the $\alpha 1$ (I), $\alpha 2$ and $\alpha 1$ (III) peptide 'maps'. The $\alpha 1$ (II) CB8, CB9.7, CB10.5 and CB11 peptides showed multiple charged forms, which were constant for different preparations (Fig. 4). The αA - and αB -chains of type V collagen produced peptide 'maps' that were distinct from those of the other collagens studied, as well as being distinct from each other (Figs. 5 and 6).

CNBr-cleavage peptides from bone

The CNBr-cleavage peptides from bone were mainly from the $\alpha 1$ (I)- and $\alpha 2$ -chains of type I collagen (Fig. 7). Peptides from type II and III collagens were not observed in normal bone samples, but heavy loadings showed a small amount of the αA and αB marker peptides.

The peptides of the $\alpha 1$ (I)- and $\alpha 2$ -chains were obtained in similar amounts from decalcified bone and purified type I collagen, except for the $\alpha 1$ (I) CB6 peptide and the minor spots of the $\alpha 1$ (I) CB3 peptide, which were recovered in lower amounts from bone. There were additional peptides located between $\alpha 1$ (I) CB6 and $\alpha 1$ (I) CB7 and between $\alpha 1$ (I) CB7 and $\alpha 2$ CB3.5 in Fig. 7 that were not observed with pepsin-solubilized collagens. The collagenous nature of this material was confirmed by

Fig. 4. Two-dimensional electrophoresis of CNBr-cleavage peptides of the $\alpha 1$ (II)-chain

A sample (200 μ g) of $\alpha 1$ (II)-chain CNBr-cleavage peptides was resolved by non-equilibrium pH-gradient gel electrophoresis as described in the text. The identities of the labelled peptides were determined by using purified peptides.

Fig. 5. Two-dimensional electrophoresis of CNBr-cleavage peptides of the αA -chain of type V collagen

A sample (50 μ g) of αA -chain CNBr-cleavage peptides was resolved by non-equilibrium pH-gradient gel electrophoresis as described in the text. The peptides marked (1) and (2) were used to identify αA -chains when other types of collagen were included in the sample.

Fig. 6. Two-dimensional electrophoresis of CNBr-cleavage peptides of the α B-chain of type V collagen

A sample (50 μ g) of α B-chain CNBr-cleavage peptides was resolved by non-equilibrium pH-gradient gel electrophoresis as described in the text. The peptides marked (1) and (2) were used to identify α B-chains when other types of collagen were included in the sample.

Fig. 7. Two-dimensional electrophoresis of CNBr-cleavage peptides of decalcified bone

A sample (200 μ g) of CNBr-cleavage peptides obtained from decalcified bone was resolved by non-equilibrium pH-gradient gel electrophoresis and SDS/polyacrylamide-gel electrophoresis as described in the text. The identities of the labelled peptides were determined by using purified peptides (see Fig. 1).

its disappearance after digestion with purified bacterial collagenase (Peterkofsky & Diegelmann, 1971). In addition, the CNBr-cleavage peptides obtained from the non-collagenous proteins of human bone, which were prepared as described by Herring (1977), did not account for any of these spots.

CNBr-cleavage peptides from dermis

The peptides obtained from insoluble dermis were derived from the α 1(I)- and α 2-chains of type I collagen and α 1(III)-chains of type III collagen (Fig. 8). Heavy loadings also showed small amounts of the α A and α B marker peptides. The dermal and bone α 1(I) CB7 and CB8 and α 2 CB4 peptides had the same number of charged forms, but the bone peptides had relatively greater amounts of the more basic components.

As with bone, the relative amounts of the α 1(I) and α 2 peptides obtained from dermis and purified type I collagen were similar, except that the amount of peptide α 1(I) CB6 was also decreased. There were also additional peptides located between peptides α 1(I) CB6 and α 1(III) CB5 and in the area marked (1) in Fig. 8. The collagenous nature of this material was also confirmed by its disappearance after digestion with purified bacterial collagenase. The relative amounts of the α 1(III) CB4, CB5 and CB8 peptides were similar in dermis and purified type III collagen, but peptide α 1(III) CB9 was absent from dermis.

Fig. 8. Two-dimensional electrophoresis of CNBr-cleavage peptides of insoluble dermis

A sample (200 μ g) of CNBr-cleavage peptides obtained from insoluble dermis was resolved by non-equilibrium pH-gradient gel electrophoresis and SDS/polyacrylamide-gel electrophoresis as described in the text. The α 1(III) and α 2 peptides and some of the α 1(I) peptides are marked. The positions of the faint α 1(I) CB3 and α 1(III) CB4 peptides are marked by circles. The peptides marked (1) represent cross-linked peptides. The other peptides can be identified by reference to Figs. 1 and 3.

Discussion

Specific 'maps' of the CNBr-cleavage peptides of human types I, II, III and V collagen chains were obtained by two-dimensional electrophoresis. The peptides showed charge heterogeneity, which was not detected when the peptides were analysed by SDS/polyacrylamide-gel electrophoresis alone. Each peptide had at least three charged forms of the same molecular weight, and this was a constant finding with different peptide preparations.

Other studies have also shown that the CNBr-cleavage peptides of collagen exist in multiple charged forms. Benya *et al.* (1980) observed similar charge heterogeneity in their two-dimensional-electrophoretic studies of peptides obtained from insoluble bone collagen. Charge heterogeneity was also evident after isoelectric focusing of $\alpha 1(I)$ peptides by Finlayson & Chrambach (1971), and Butler *et al.* (1967) observed that the $\alpha 1(I)$ CB6, CB7 and CB8 peptides were heterogeneous, as each peptide could be resolved into several components by rechromatography on CM-cellulose. This was confirmed in the present study when the $\alpha 1(I)$ CB7 and CB8 peptides were rechromatographed, and analysis of these components by two-dimensional electrophoresis showed that the peptides eluted first were more acidic than those eluted later. Similar elution profiles were observed by Chung *et al.* (1974) when the $\alpha 1(III)$ CB3, CB4, CB5, CB6, CB7 and CB8 peptides obtained from human dermal type III collagen were rechromatographed.

The charge heterogeneity was considered to indicate that each collagen chain existed in several forms as the result of translational and/or post-translational differences. This view is supported by the reproducible nature of the charge heterogeneity with different peptide preparations and by our finding that uncleaved α chains also showed charge heterogeneity on two-dimensional electrophoresis (results not shown). To what extent translational differences accounted for the observed charge heterogeneity is uncertain, but Butler *et al.* (1977) have shown that amino acid sequence heterogeneity exists in the $\alpha 1(II)$ -chains of bovine nasal cartilage. Sequence heterogeneity also occurs between tissues, as reported by Seyer *et al.* (1980) for type III collagens from human liver and dermis. Tissue heterogeneity of this type may account for some of the differences in charge heterogeneity noted in the present study between the type I collagens of bone and dermis. This mechanism was also suggested as an explanation for the peptide-'map' differences observed between corneal and tendon type I collagens by Kao & Foreman (1980).

However, it is more likely that the charge heterogeneity was due to variations in the post-translational modifications of the collagens, as

variations in the extent of prolyl and lysyl hydroxylation and glycosylation have been observed during amino acid sequence studies (Fietzek & Kuhn, 1976). Differences in the extent of lysyl hydroxylation and glycosylation have also been observed in type I collagens obtained from different tissues (Fietzek & Kuhn, 1976). This type of microheterogeneity may also account for the differences noted between the two-dimensional peptide 'maps' of the type I collagens of dermis and bone.

Chemical modifications of the collagen peptides during CNBr cleavage and during the electrophoretic procedures also need to be considered as possible sources of charge heterogeneity. However, several lines of evidence suggest that these mechanisms were not responsible for the charge heterogeneity observed in this study.

During the CNBr cleavage, methionine is converted into homoserine and homoserine lactone, but as recommended by Finlayson & Chrambach (1971), homoserine lactone was converted into homoserine with NaOH before electrophoresis. The presence of two forms of homoserine is therefore unlikely to be the cause of the charge heterogeneity, as the heterogeneity persisted after the pH was raised. In addition, the C-terminal $\alpha 1(I)$ CB6 and $\alpha 1(III)$ CB9 peptides showed charge heterogeneity although they do not contain homoserine (Butler *et al.*, 1967; Chung *et al.*, 1974).

Deamidation of asparagine and glutamine residues in peptides of type III collagen was noted by Lang *et al.* (1979) during their amino acid sequence studies. However, they suggested that deamidation had occurred during the treatment of the CNBr-cleavage peptides with hydroxylamine, and in the sequencer rather than during the initial CNBr cleavage. However, as the CNBr reaction is carried out in 70% formic acid it is possible that deamidation may also occur during this procedure. It has also been reported that spontaneous deamidation of these residues may occur when proteins are stored for even short periods (O'Farrell, 1975), but we did not observe any electrophoretic changes with storage of the collagens or their peptides. O'Farrell (1975) and Wallevik (1973) have reported that charge heterogeneity may be produced as an artifact by chemical modifications or by the formation of Ampholine-peptide complexes during sample preparation for electrophoresis. These mechanisms were not considered to be the causes of the charge heterogeneity observed in the present study as the $\alpha 1(I)$ CB7 and CB8 peptides were shown by rechromatography on CM-cellulose to contain multiple charged forms before the peptides were prepared for electrophoresis.

The CNBr-cleavage peptides obtained directly from insoluble dermis and decalcified bone showed the same type of charge heterogeneity as was

observed with the peptides from solubilized collagens. The peptide 'maps' were reproducible and were readily obtained with small samples of tissue. It was also possible to study the involvement of peptides in cross-linkages. In agreement with Light & Bailey (1979), the formation of collagen cross-linkages did not appear to involve, to any major extent, the $\alpha 1(I)$ CB3, CB7 or CB8 peptides, and this also applied to the $\alpha 1(III)$ CB4, CB5 and CB8 peptides. However, the $\alpha 1(III)$ CB9 peptide was absent from all dermal samples, and peptide $\alpha 1(I)$ CB6 was decreased in bone and dermis, probably owing to their involvement in acid-stable cross-linkages (Light & Bailey, 1979; Nicholls & Bailey, 1980). The two-dimensional 'maps' also contained some additional peptides that could not be identified from the peptide 'maps' obtained with pepsin-solubilized collagens. They were probably cross-linked peptides containing the $\alpha 1(I)$ CB6 peptide cross-linked to the small $\alpha 1(I)$ CB1 and $\alpha 1(I)$ CB5 peptides described by Light & Bailey (1979). Benya *et al.* (1980) from their two-dimensional-electrophoresis studies of cross-linked peptides of insoluble bone collagen also suggested that these additional peptides contained the $\alpha 1(I)$ CB6 peptide cross-linked to other small peptides.

This approach to the study of the heterogeneity of human collagens has several advantages for the investigation of human connective-tissue diseases. It is suitable for the analysis of small samples and enables an assessment to be made of charge heterogeneity, types of collagen and some aspects of cross-linkages. The gels were easy to compare, although the comparisons were qualitative, but computer methods are available for quantitative analysis (Garrels, 1979).

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After this paper was submitted for publication Benya (1981) published a paper describing the resolution of types I, II and III collagens by non-equilibrium pH-gradient gel electrophoresis.

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