Ehrlich chromogens, probable cross-links in elastin and collagen

Paul D. KEMP and John E. SCOTT*

Chemical Morphology, Cell and Structural Biology, Chemistry Building, University of Manchester, Manchester M13 9PL, U.K.

(1) Proteolytic digests of tissue elastin contain material which reacts with dimethylaminobenzaldehyde in acid solution (Ehrlich's reagent) to give a cherry-pink colour. This Ehrlich chromogen(s) [EC(s)] is similar to but not identical with EC(s) previously demonstrated in tissue collagens [Scott, Hughes & Shuttleworth (1979) *Biosci. Rep.* 1, 611–618]. Both ECs react with diazonium salts in acid to give coloured products. (2) Diazobenzene linked via a phenolic ester to polyacrylamide beads (Biogel P10) has been used to absorb ECs specifically and almost quantitatively from proteolytic digests. The coupled deeply coloured azo-EC-peptides were then recovered after mild alkaline cleavage from the support and purified by gel chromatography. (3) Using ^{15}N -labelled NaNO_2 , the collagen azo-EC-peptides were prepared, and ^{15}N abundance measured therein. The molar absorption coefficient of the azo-EC group was calculated (18 700) based on the assumption that each azo-EC group contained one ^{15}N atom. (4) Collagen azo-EC-peptides contained glucose and galactose, whereas elastin azo-EC peptides did not. The amino acid patterns of the two peptides were quite different, the former being rich in polar amino acids, the latter containing much alanine. The patterns were compatible with an origin from the cross-linking regions of collagen and elastin respectively. (5) Quantitative (molar) comparisons of the azo-EC group content with amino acid, amino end-group and sugar contents, and azo-EC peptide molecular mass, suggest that a structure is present in the collagen azo-EC-peptides containing two EC groups shared between four peptide chains. Three peptide chains probably meet at each (cross-linking) EC group. (6) Based on this structure, about 15% of adult bovine skin collagen contains EC groups.

INTRODUCTION

Collagen was shown to contain material that reacted with *p*-dimethylaminobenzaldehyde in acid solution (Ehrlich's reagent) to give a cherry-pink colour [1]. The Ehrlich chromogen (EC) also reacted with aryldiazonium salts at pH 1–2 to give coloured products [2]. These properties, together with the acid lability of EC and its non-reducibility by borohydride, implied that a nitrogen heterocycle, possibly pyrrolic, was involved [1]. EC was not present in salt-soluble collagen, but was abundant in polymeric collagen. It was therefore suggested that EC was a cross-linking species, and this was strongly supported by the observation that a rich source of EC was a three-chain peptide of 50 residues derived from the cross-link region of type III collagen [3]. An EC was also found in the 7S region of type IV collagen [3]. Pyridinoline, a pyridinium compound present in collagen as a cross-link, was not an EC [4].

An affinity-support method for recovering peptide-linked EC from papain or pronase digests of connective tissue was developed, based on the acid diazonium-coupling reaction [2]. Cellulose was the support, to which diazobenzene was linked via an amide bond. EC was quantitatively absorbed from digests, as a yellow azo derivative, leaving > 99% of the other peptides in the supernatant. The yellow azo-EC-peptides were then cleaved from the support in hot NaOH. This approach had shortcomings; the recovery of colour was low, and sugars in the azo-EC-peptides could have come from the cellulose, although there were indications that some of them did not. A new support has therefore been

developed, utilizing a polyacrylamide substrate to which a diazobenzene group is linked via an alkali-labile phenol ester. The derived yellow azo-EC-peptides have been recovered in high yield, and characterization of the peptides has been greatly facilitated.

Cross-links in elastin (the pyridines desmosine and isodesmosine) originate from enzyme-catalysed oxidative deamination of lysine residues by reactions similar to those giving rise to collagen cross-links, some of which also develop into pyridinium derivatives (pyridinoline and 3-deoxypyridinoline [4] [5]). If the EC(s) in collagen are indeed cross-links derived from oxidized lysine and hydroxylysine, it was of great interest to see whether the parallels between collagen and elastin extended to the presence of EC(s) in the latter. This paper shows that there were indeed EC(s) in elastin. Using the improved affinity support to isolate them, elastin azo-EC-peptides have been compared with those from collagen.

Some of these results have been preliminarily communicated [5].

EXPERIMENTAL

Materials

Biogel P10 beads were from Bio-rad Laboratories (Watford, U.K.). Cellulose acetate membranes for electrophoresis were from Sartorius. ^{15}N -labelled NaNO_2 was from Amersham International (Aylesbury, U.K.), Batch no. BA5, chemical purity 98%, isotopic abundance 91.4%. Armeen M2C (methyldidodecylamine) was from Azco Chemicals. Enzymes were from

Abbreviations used: EC, Ehrlich chromogen; PTC, phenylthiocarbamyl; PTH, phenylthiohydantoin.

* To whom correspondence and reprint requests should be addressed.

Sigma Chemical Co. Other chemicals were commercial, best obtainable, grades. Bovine skin and ligamentum nuchae were obtained fresh from an abattoir, freed of adventitious tissue or hair, and chopped into small pieces. Skin was degreased and dehydrated in a series of ether/ethanol solutions (0:100, 1:3, 1:1, 3:1, 100:0%, v/v). Ligamentum nuchae was used without chemical cleaning. An estimate of the moisture content was derived from the weight loss on freeze drying to constant weight.

Methods

ECs were measured by reaction in acid solution with *p*-dimethylamino benzaldehyde [1]. Hydrolysis of peptides or proteolytic digests for amino acid assays was in 6 M-HCl at 105 °C for 16 h. Deacidification was by extraction with 0.5 M-methyldidodecylamine in chloroform or by repeated evaporation *in vacuo*. Sugars were liberated from azo-EC-peptides by hydrolysis in 0.5 M-H₂SO₄ at 100 °C for 1 h, and de-acidified as above. Hydroxyproline was assayed according to Woessner [6]. Ammonia was determined according to Fawcett & Scott [7]. Amino acids analyses were performed at the Max Planck Institut für Biochemie, Martinsreid, Munich, Germany, using a Biotronic LT 5001 amino acid analyser.

Amino acid paper chromatograms. These were run in *n*-butanol/acetic acid/water (12:3:5, by vol.) either directly or after electrophoresing the sample in pyridine acetate buffer (0.45% pyridine/0.26% acetic acid in water) at right angles to the chromatographic run. The paper was either dried on a glass plate at 90 °C, or freeze-dried overnight after electrophoresis but before chromatography. Staining was with 1.0% ninhydrin in acetone containing about 1.0% pyridine.

Sugar. Chromatography was on Whatman No. 54 paper in ethyl acetate/pyridine/water (12:5:4, by vol.) after preliminary electrophoresis in pyridine acetate buffer as described above. Staining was with AgNO₃ [8].

¹⁵N isotopic abundance. Analyses were obtained on solid samples using an AWA 1500 nitrogen analyser (Carlo Erba Strumentazione, Milan, Italy) connected to a VG Isogas triple collector SIRA Mass spectrometer (V.G. Isogas, Middlewich, U.K.).

CHN analyses. These were by Mr. M. Hart, Analytical Laboratory, Chemistry Department, University of Manchester, U.K.

Phenylthiocarbamyl (PTC) derivatives. Peptides or amino acids in water (1 vol.) were mixed with phenylisothiocyanate/triethylamine/pyridine (1:1:18, by vol.) (2 vol.) and incubated at 45 °C for 45 min. The solution was extracted 3 times with 3 vol. of benzene, and then 2 times with 3 vol. of diethyl ether. Samples were dried by rotary evaporation *in vacuo* at 50 °C. PTCs in ethanol were quantified from their absorption at 240 nm using a molar absorption of 16000 [9].

Phenylthiohydantoin (PTH) derivatives. Dry PTC derivatives, prepared as above, were converted into PTH derivatives via their thiazolinones. The PTC compounds were incubated at 30 °C in trifluoroacetic acid for 30 min,

the trifluoroacetic acid was removed under vacuum, the thiazolinones were extracted into ethyl acetate and dried, and were then converted into PTH derivatives by incubation at 32 °C in 3 M-HCl for 30 min. PTHs were extracted into ethyl acetate, dried, and quantified by their absorbance at 270 nm in ethanol [9].

Periodate oxidation and electrophoresis in bisulphite buffer. Sodium periodate (0.2 M, 2 µl) was added to a small amount of freeze-dried peptide in a stoppered tube and left in the dark for 15 min. Sodium metabisulphite (1.1 M, 1 µl) was then added and left for a further 15 min. The sample was applied to cellulose acetate membranes as an 0.5 cm streak and electrophoresed at 10 V/cm in 0.025 M-sodium metabisulphite, pH 4.3. For the control, 1 vol. of 0.2 M-sodium periodate reacted with 2 vol. of 1.1 M-sodium metabisulphite for 15 min and 3 µl of this solution used to dissolve the peptide.

Gel chromatography. This was carried out on Biogel P10 beads in jacketed columns cooled with running tap water. The eluting solvent was 1% (w/v) ammonium acetate. Fractions were collected automatically. Absorbances were measured manually. The excluded volume (*v*₀) was determined using human haemoglobin.

Ultracentrifugation. Ultracentrifugation of azo-EC-peptide was carried out at 20 °C in a Beckman L87C ultracentrifuge, scanning at 280 nm.

Preparation of diazotized support. The hydrazide derivative of polyacrylamide was made according to Inman [10]. To 10 g of Biogel P10 beads were added 125 ml of water and 25 ml of 40% (v/v) solution of hydrazine hydrate. The slurry was mixed at room temperature for 48 h, collected by filtration under suction, and washed with 0.2 M-NaCl until the effluent produced a negative reaction for amines with trinitro-benzenesulphonic acid [10].

Subsequent reactions were at 4 °C in the dark. To the gel hydrazide was added 900 ml of 0.2 M-HCl, plus 90 ml of 4% (w/v) NaNO₂, with stirring for 15 min. The gel was collected by filtration and washed with 1000 ml of water. Excess HNO₂ was removed by stirring in 450 ml of 1% (w/v) ammonium sulphamate. The gel was collected by filtration and washed with 1000 ml of water.

The gel azide was mixed with stirring in 1000 ml of 2% (w/v) nitrophenol in 0.15 M-sodium carbonate buffer, pH 8.7, for 1 h. Excess nitrophenol was removed by washing the gel successively with 4000 ml each of 0.15 M-sodium carbonate buffer, pH 8.8, water, and 0.6 M-sodium bicarbonate.

Reduction of the aryl nitro group took place in 100 ml of 10% (w/v) aqueous sodium dithionite/0.6 M-sodium bicarbonate for 1 h and the gel was washed with 4000 ml of water. The gel was stored at -20 °C.

Diazotization took place in 1000 ml of 0.1 M-HCl/20 ml of 4% NaNO₂ for 15 min with stirring; the gel was then washed with 2500 ml of 0.04 M-HCl, treated with 200 ml of 1% ammonium sulphamate in 0.04 M-HCl for 5 min and washed with 5000 ml of 0.04 M-HCl.

Preparation of azo-EC-peptides (PAP 18). Dried degreased skin (50 g) was digested for 6 h with 20 mg of crystallized papain at 65 °C in 125 ml of solution

Fig. 1. Elution profiles on Biogel P10 (200–300 mesh) beads of azp-EC-peptides from papain-digested bovine skin (PAP 18)

For details see the text. Full line, ^{14}N peptides prepared using unlabelled NaNO_2 ; broken line, ^{15}N peptides labelled in the diazo ($-\text{N}=\text{N}-$) group with ^{15}N . The figures inside the ordinate and abscissa refer to the ^{15}N peptides, those outside refer to the ^{14}N peptides. V_e for this column was 30 ml. The peptides were cleaved from the polyacrylamide support, and the contaminating naphtholazophenates removed by n-pentanol extraction before applying to the P10 column. The elution profile of the ^{14}N peptides was obtained using this material, the peak (around 69 ml) corresponding to a molecular mass of about 3500 Da. The smaller peak at about 130 ml contained contaminating naphtholazophenates. ^{15}N peptides prepared in parallel at the same time gave a very similar profile. The main peak (i.e. minus the smaller contaminant peaks at around 109 and 130 ml) of the ^{15}N peptides was taken, dialysed for 24 h, and re-applied to the column to give the elution profile shown by the broken line. The main peak, at around 53 ml, has a molecular mass of about 4400 Da. This material was used to obtain the spectroscopic and chemical data reported in the text.

containing 0.3 M-NaCl/5 mM-disodium EDTA/5 mM-cysteine hydrochloride and 0.1 M-sodium phosphate buffer, pH 6.4 [11]. A further 20 mg of papain was added and digestion continued overnight. Finally the temperature was raised to 80 °C for 3 h. The digest was centrifuged and hair and deposits were removed. To the brown viscous solution (145 ml) was added 7.5 ml of 5% (w/v) aqueous cetyltrimethylammonium bromide (to precipitate polyanions [1]) and 290 ml of water. The solution was left at 4 °C overnight, centrifuged, and to 340 ml of clear supernatant (equivalent to about 40 g of skin) was added 9.9 ml of conc. HCl to a pH of 1.3. Subsequent operations were performed at 4 °C in the dark. Freshly diazotized gel (12.5 g) (in this case using *p*-nitrophenyl and ^{15}N labelled NaNO_2 , see above) was added to the cooled acidified digest, and left for 1 h with stirring. The gel, which had turned a deep orange-brown, was filtered, washed twice with 240 ml of 0.1 M-HCl, then four times with 240 ml of 0.04 M-HCl. α -Naphthol [120 ml, 1.2% (w/v)] in 33% (v/v) ethanol/water was added to the gel with stirring for 5 min followed by 120 ml of 1.2% (w/v) α -naphthol in aqueous 0.5 M-sodium acetate solution, and left for 55 min. The gel was filtered, washed ten times with 100 ml of 30% (v/v) ethanol/water, and treated in a test-tube with 7 ml of

30% (v/v) 0.880 ammonia solution ($\sim 5\text{ M-NH}_4\text{OH}$) at 40 °C for 20 min. The gel was filtered and washed with 5 M-ammonia solution until the filtrate was colourless. The filtrates, which totalled 43 ml, were evaporated to dryness under reduced pressure at less than 50 °C. The solid was partitioned between 10 ml of water and 10 ml of water-saturated n-pentanol, replacing the n-pentanol layer 5 times, and pooling the organic phases.

The aqueous phase was concentrated, applied to a P10 Biogel column, and eluted with 1% (w/v) ammonium acetate solution. The greater part of the colour (80% estimated at 400 nm) eluted in a broad band with a molecular mass corresponding to 1000–5000 (Fig. 1). A second, much smaller, peak was clearly separated, running in a position corresponding to a very low (< 0) molecular mass (i.e. it was chromatographing by absorption rather than by gel filtration). The pooled main peak was concentrated under reduced pressure, freeze-dried, dissolved in 1 ml of water, and then dialysed for 6 h at 4 °C against three changes each of 2 l of distilled water to remove remaining ammonium acetate, as monitored by NH_3 assays on this dialysate. Colour (54.1%) was recovered after dialysis. This material eluted from a P10 Biogel column as a single broad peak with a V_e/V_0 corresponding to a molecular mass of 4.4 kDa. It was submitted to CHN and ^{15}N analyses.

The n-pentanol extracts containing ^{15}N -labelled α -naphthol-azophenol was washed twice with water, and evaporated to dryness under reduced pressure. The solid was extracted with ethanol/water (4:1, v/v), the supernatant was evaporated to dryness, and the resulting solid used for CHN and ^{15}N analyses

RESULTS

Proteolytic digests of ligamentum nuchae (mainly elastin), as well as skin (mainly collagen), contained EC peptides. Elastase liberated large amounts of EC from ligamentum nuchae without dissolving the entire tissue. Papain digestion at 60 °C completely dissolved ligamentum nuchae in a few hours, liberating over twice as many colour units/g of Ehrlich reactivity as that from dry defatted skin, and over four times as much azo-EC colour/g.

The EC from ligamentum nuchae was much more sensitive to acid than that from skin and bone. Less than 20% of the reactivity towards Ehrlich reagent remained after 30 min at room temperature in 5 M-HCl, whereas after 3 h, skin or bone papain digests had lost no reactivity.

The Ehrlich product from ligamentum nuchae had a slightly different spectrum to that of skin (λ_{max} , 566 nm and 572 nm, respectively).

Diazotized *m*- and *p*-phenols, ester-coupled to Biogel P10 polyacrylamide beads, removed almost all EC from acidified papain digests of bovine skin and ligamentum nuchae, turning a deep orange-brown colour. Unreacted diazonium groups were coupled to α -naphthol. The coloured products were quantitatively eluted from the beads at 40 °C in 5 M- NH_4OH . The deep-red α -naphthol-azo-phenols were removed by extraction into n-pentanol, leaving azo-EC-peptides in the aqueous phase. The latter were further purified by gel chromatography on Biogel P10 beads. The greater part of the colour appeared as a broad band (Fig. 1). The freeze-dried material represented much less than 1% of the

Table 1. Amino acid composition (residues/1000) of azo-EC-peptides from bovine skin and ligamentum nuchae

Bovine skin amino acids are mainly collagen-derived, while the ligamentum nuchae amino acids are mainly from elastin.

Amino acid	Composition	
	Bovine skin	Bovine ligamentum nuchae
Hyp	56.2	6.8
Asp	83.0	3.7
Thr	28.6	10.9
Ser	43.6	16.7
Glu	121.4	12.5
Pro	85.8	147.0
Gly	244.6	290.1
Ala	51.6	227.9
Cys	—	4.4
Val	17.6	114.6
Met	14.3	—
Ile	17.9	28.6
Leu	35.1	46.1
Tyr	45.1	20.4
Phe	23.2	41.9
Hyl	20.1	—
Lys	16.4	—
His	37.2	28.5
Arg	58.3	—

total protein. Control experiments, minus only the tissue, yielded insignificant amounts of coloured material in this fraction.

The product was heterogeneous, as shown by (1) a broad P10 elution curve, (2) multiple banding and streaking on paper chromatograph in butanol/pyridine/water, phenol/water, n-butanol/acetic acid/water, and (3) electrophoresis in a variety of buffers, on paper and cellulose acetate, in which the coloured products ran as streaks with no obvious banding patterns.

Re-digestion with papain, ficin, Pronase, trypsin, chymotrypsin, elastin or collagenase did not reduce the heterogeneity on P10 chromatography, and although the molecular mass was decreased by some of these treatments, it remained above 3000 kDa. Digestion with carboxypeptidase likewise had little effect on the molecular mass, and only small amounts of amino acids were liberated.

Analyses

Quantities of EC-peptide are expressed as 'colour units', i.e. absorbance of a solution in a 1 cm cuvette, multiplied by the total volume of the solution.

Elemental analyses (including ^{15}N). PAP 18 azo-EC-peptide contained 44.85% C, 6.55% H and 14.1% N. ^{15}N constituted 3.72 (± 0.08)% of the total N. The naphthol-azo-*p*-phenate dye isolated simultaneously with PAP 18 azo-EC-peptides contained 70.7% C, 5.2% H and 9.3% N. The theoretical values for an α -naphthol-diazo-phenol are 73% C, 4.5% H and 10.6% N. ^{15}N constituted 43.14 (± 0.8)% of the total N. Correcting for natural abundance of ^{15}N (0.366%), the ^{14}N enrichment of the azodye is close to the theoretical,

assuming 2 N atoms/mol, and taking the ^{15}N abundance of the $\text{Na}^{15}\text{NO}_2$ as 91.5%.

Using these data, the ^{15}N enrichments of PAP 18 azo-EC-peptides was 3.9%.

Amino acids and end groups. Table 1 shows the composition of azo-EC-peptides from bovine skin and ligamentum nuchae. Both contain large amounts of glycine. Glutamic acid is dominant in the skin peptides, but almost absent from ligamentum nuchae material, which, conversely, is far richer in alanine and valine.

At least seven different PTH end groups were found in each 'collagen' preparation, in varying amounts. Glycine, alanine, glutamic acid and tyrosine were usually present in the largest amounts.

PAP 18 contained 0.11 μmol of NH_2 endgroups/colour unit by the PTC method, and 0.112 μmol /colour unit by PTH estimation. Total amino acids after hydrolysis were 0.95 μmol /colour unit, by the PTC method, and 0.6 μmol /colour unit by the PTH method. The former figure may be too high, because of phenylthiourea formation from non-amino acid compounds, and the latter too low because some amino acids (arginine, serine, threonine) are not fully recovered, or give atypical u.v. spectra.

Molecular masses

Approximate molecular masses were determined from the V_e/V_0 graph of Biogel P10 beads, provided by the manufacturers. The peak of PAP 18 (Fig. 1) corresponded to 4400 kDa. Another preparation, PAP 13, which had been extensively dialysed, gave a peak corresponding to 5900 kDa. This estimate was confirmed by ultracentrifugation, which gave a sedimentation coefficient of 0.72 (± 0.017) $\times 10^{-13}$ s, corresponding to a molecular mass of 5000–7000, for a normal peptide. These are average figures, referring to a broad spread of molecular masses.

The molecular mass of PAP 18 was also estimated from the ^{15}N content. If one molecule of PAP 18 azo-EC-peptide contains one atom of ^{15}N , and ^{15}N enrichment is 3.9%, then the total number of N atoms/molecule is 100/3.9, i.e. 25.64, weighing 359 Da. This is 14.1% of the whole molecule, which therefore weighs 2550 Da. This figure is compatible with the amino acid analyses. Fifteen (average of PTC and PTH analyses) amino acid residues (about 1500 Da), weighted by the large proportion of glycine (Table 1), one residue of azo-phenol (120 Da), plus sugars (250–350 Da), add up to about 1900 Da. The contribution of EC, if it were of a size similar to pyridinoline or desmosine would account for the remainder.

The elastin EC peptides were smaller (< 2100 Da by P10 chromatography) than the collagen EC peptides.

Spectrophotometry of the azo-EC-peptides

Both *m*- and *p*-phenol-azo-EC-peptides from collagen had peak absorptions at around 400 nm. The *para* derivative had a shoulder at about 490 nm, which was missing from the *meta* compound. This shoulder developed at the expense of the main (400 nm) peak on acidification, until it was the only peak. The *meta* dye behaved similarly, but the spectrum was much less sensitive to pH change; the longwave shoulder (around 450 nm) was clearly visible only below pH 2.0. The

spectra of *meta* and *para* derivatives were not affected by pH changes in the region of pH 5–10.

An estimate of the molar absorption of the *p*-phenol-azo-EC group was based on the ^{15}N -labelled PAP 18 preparation, assuming that each azo ($-\text{N}=\text{N}-$) group contained one ^{15}N atom derived from $\text{Na}^{15}\text{NO}_2$. A solution of PAP 18 at 1.13 mg/ml, containing 14.1% N, enriched by 3.9% ^{15}N , read $A_{1\text{ cm}, 400}$ 7.8. The percentage of ^{15}N by weight is $3.9\% \times 14.1\%$ (0.55%). In 1.13 mg of PAP 18 there were therefore 6.25 μg of ^{15}N /mol. The molar absorption of the azo dye was therefore 18700.

^{15}N data on *m*-phenol derivatives were not obtained, but colour yields from tissues processed in parallel to produce *para* and *meta* derivatives were similar, and to a rough approximation the molecular absorptions are likely to be the same.

Tissue concentrations of EC

Six preparations of *p*-azo-EC-peptides from five skin samples yielded 3.52 ($\pm 20\%$) colour units/g of dried defatted skin, which contained 84 mg/g of Hyp (i.e. about 80% collagen, molecular mass 3×10^5 Da). Thus, 0.071 mol of azo-EC was present per mol of collagen.

Ligamentum nuchae azo-EC-peptides yielded 22.5 units/g dry wt., after complete digestion at 60 °C. The Hyp concentration was 2.19%, equivalent to 20% collagen. The yield in the Ehrlich reaction was 11.5 units/g.

Comparison of Ehrlich reaction with diazonium coupling and elution

The five samples which yielded 3.52 azo-EC colour units/g of skin gave 5.53 ($\pm 22\%$) units/g in the Ehrlich reaction. The Ehrlich assay, performed before the coupling reaction, includes reactants which were not coupled ($< 10\%$ of the total). The molar absorption of the Ehrlich reaction is therefore a little higher than that of the diazonium reaction, in the region 20000–25000, compared with 18700.

The ratio of Ehrlich to diazo colour units yielded by ligamentum nuchae was only 0.5, compared with > 1.0 for the collagen material.

Sugars

Glucose and galactose were found in approximately equal amounts in 0.5 M- H_2SO_4 hydrolysates of skin diazo-EC-peptides, but no carbohydrate was detected in the ligamentum nuchae peptides.

The periodate-oxidized collagen azo-EC-peptide gave a purple Schiff reaction with λ_{max} at 561 nm.

After oxidation with periodate, electrophoresis in metabisulphite buffer at pH 4.3 showed that at least 50% of the colour travelled more rapidly (as an anion) than control azo-EC-peptide which had not been oxidized. Periodate oxidation released about 1 μmol of acid/ μmol of colour.

By comparison with standard spots, about 0.005 μmol of glucose or galactose/colour unit was present in acid hydrolyses of collagen *m*-azo-EC-peptide.

Summary

Thus, based on a molar absorption of 18700, the following molar ratios per azo-EC group are calculated:

PTC end groups 2.06; PTH end groups 2.1; amino-acid content (PTC) 17.8; amino-acid content (PTH) 11.1; sugar (Ag stain, HCl hydrolysis) 0.1–0.2; sugar

(titration after periodate) 1; azo-EC-peptide (4400 molecular mass) 0.57. In view of the broad gel-chromatography peak, some of these figures (particularly the amino acid contents) are probably averages.

DISCUSSION

The characteristic distribution of EC(s) in collagen and derived peptides pointed to them being cross-linking species [1,3]. Underivatized ECs cannot be isolated and identified by the standard methods used for other cross-links. They are rapidly destroyed by hot acid, and, being non-reducible by borohydride they cannot take up tritium label from borotritide as some other cross-links do. For these reasons, an affinity support was devised, utilizing the fairly specific diazo-coupling reaction of EC at low pH, which, it was hoped, would provide high yields of EC-peptides, relatively uncontaminated by other peptides, with a coloured label attached directly to the EC group. The first attempt [2], based on diazobenzamidocellulose, was successful in that EC was quantitatively and specifically bound to the support and the amino acid patterns of the bound material were clearly collagenous, but recovery from the support was low. Cleavage of the benzamide required fairly drastic conditions (2 M-NaOH at 100 °C). Under these conditions the cellulose support itself liberated materials which seriously contaminated the products. We therefore developed an improved affinity support, based on polyacrylamide, to which a diazonium phenol ester was linked. The phenol azo-EC-peptide ester was quantitatively cleaved in mild conditions, to yield yellow azo-EC-peptides, with no contamination from by-products of the support. A considerable proportion of the diazonium groups remained unreacted after EC-peptides had coupled, and these were 'blocked' with α -naphthol, to give deep-red azo-naphthol-phenol dyes, which were easily separated from the azo-EC-peptides by *n*-pentanol extraction. This procedure represents a further improvement over the previous method, in which the unreacted diazonium groups were converted into azides. Aromatic azides can photoreact to form free radicals [12] which in turn may stimulate cross-linking and other side reactions inimical to unambiguous analyses and conclusions. We carried out all stages involving active diazonium groups in the dark.

Two versions of the diazonium phenol-ester-polyacrylamide support have been investigated. The first, based on *p*-aminophenol, was satisfactory in almost all respects except that the azo-EC-peptides were found to break down solely in the very specific conditions ($\sim$ pH 8.0, in solution containing Mn^{2+} ions) necessary for prolidase activity. This was traced to loss of *p*-diazoniumphenate, a quinone diazide [13], and hence an excellent leaving group. To avoid such complications, the second version was based on *m*-aminophenol, which, having no quinonoid properties, is stable under the conditions in which the azo-*p*-phenol broke down. This support has been used in all later experiments, including those with elastin peptides.

By labelling the diazonium group with one atom of ^{15}N per mol via HNO_2 at the diazotization stage, it was possible to quantify the azo-EC colour in molar terms, assuming one azo ($-\text{N}=\text{N}-$) group/azo-EC group. Uptake of ^{15}N was checked by parallel and simultaneous labelling of α -naphthol-azo-phenol attached to the same

support. The molar absorption was calculated as 18 700 per azo-EC group (see the Results section).

The molar ratio of PTH or PTC amino end groups to azo-EC groups in PAP 18 was then very close to 2.0. In other less-well-characterized azo-EC-peptides this ratio varied, but it was always considerably higher than 1.0, and sometimes over 2.

The molecular mass of the peptide PAP 18, based on one azo-EC group/mol of peptide, is about 2500 (see the Results section), and this is compatible with the amino acid composition (see the Results section). The molecular mass of PAP 18 from the Biogel P10 column is much higher than this, by a factor of about 2 (see the Results section), suggesting that there are two azo-EC groups/mol of PAP 18. The simplest structure to account for the data is one in which each of the two azo-EC groups link three polypeptide chains, overlapping to give 4 chains per 2 EC units. The EC groups are assumed to be linked by short stretches of peptide which are difficult for exoproteases to digest.

Sugars

Collagen-derived azo-EC-peptides contained glucose and galactose liberated in approximately equal amounts by acid hydrolysis. Oxidation with periodate produced a Schiff-reactive material and caused liberation of titratable acidity. The aldehydes produced were converted in metabisulphite solution into hydroxyalkane sulphonic acids and electrophoresed in bisulphite buffer [14,15]. The oxidized azo-EC-peptide colour moved much more rapidly as an anion in bisulphite solution at pH 4.3 than the control (non-oxidized) colour, proving that a large part of the azo-EC-peptide was covalently associated with periodate oxidizable material.

Glycoside may be attached to each EC unit, based on the titratable acid liberated by periodate oxidation (see the Results section), assuming that the acid was formic acid. Formic acid (1 mol) would be produced from either a monosaccharide (galactose) or disaccharide (glycosylgalactose), assuming a similar structure to that present in other collagen-linked glycosides [16,17]. The presence of both glucose and galactose in PAP 18 azo-EC-peptide indicates that at least some of these glycosides are disaccharides.

Elastin ECs

The small amount of collagen in ligamentum nuchae is quite insufficient to account for the large amount of EC present. The demonstration that elastic contains ECs, isolatable as peptides by methods identical with those used to prepare collagen azo-EC-peptides, throws additional light on the origins and functions of ECs. Cross-links in both proteins originate from oxidized lysine residues. Succeeding stages must be similar, but not identical, since some of the 'mature' cross-links differ in detail (desmosines as compared with pyridinolines). In fact, some properties of elastin ECs are different from those of collagen ECs. The former are more acid labile, and the spectra of their products with Ehrlich's reagent are slightly different. The ratio of colour produced in the Ehrlich reaction to that isolated from the diazonium support is much less in the case of elastin ECs.

Elastin azo-EC-peptides contained no detectable carbohydrate, in contrast with those from collagen.

ECs as cross-links

The discovery that a well-characterized cross-linked three-chain polypeptide from type III collagen contained considerable amounts of EC reinforced earlier ideas that EC was a cross-link [3]. An homologous cross-linked two-chain polypeptide did not contain EC, suggesting that EC was a trifunctional compound. The amino acid compositions of EC-peptides absorbed to an affinity support implied that they were derived from parts of the collagen molecule known to be involved in cross-linking [2]. For reasons discussed above, unambiguous interpretations of these results was difficult. Preparations of azo-EC-peptides from the new supports provide much clearer data. In particular, the evidence shows that ECs are associated with more than one polypeptide chain at a time.

The amino acid pattern of skin azo-EC-peptide (Table 1) is undoubtedly collagenous. Considerable amounts of hydroxyproline and a high content of glycine suggest that helical regions contribute to the EC-peptides. Tyrosine (present only in telopeptides), and histidine, methionine and hydroxylysine (present almost exclusively in helical regions close to residues 87 and 920 in types I and III collagen which are involved in cross-link formation) are important marker amino acids, much enriched in azo-EC-peptides. The presence of glucose and galactose also suggests the involvement of cross-linking regions [17]. These analyses demonstrate that the environment of EC is typical of telopeptide-helical region cross-links.

Elastin azo-EC-peptide amino acid patterns differ radically from those of collagen azo-EC-peptides (Table 1). The high alanine level is compatible with the presence of some cross-linking regions of elastin [18].

Tissue contents of ECs

The ^{15}N -based estimate of the molar absorption of the azo-EC implies that for each mol of tissue collagen there is 0.071 mol of EC (see the Results section). This estimate is similar to that published earlier [1], but the resemblance may be fortuitous; the earlier estimate was based only on spectral analogues of the Ehrlich product with model compounds, and there was no information at that time about possible overlapping structures. Nevertheless, the comparison of diazo with Ehrlich colours in this paper (see the Results section) suggests that the Ehrlich reaction provides a simple and acceptable estimate of tissue concentrations of collagen EC assuming a molar absorption of 20 000–25 000.

The much higher yield of Ehrlich colour/g of dry elastin implies that elastin is a richer source of EC than collagen. Since the elastin EC(s) structure differs from that of collagen EC(s), and no ^{15}N -based estimate of the molar absorption of elastin azo-EC is available, a reliable calculation of elastin EC tissue concentration is not yet possible.

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