Separation of a Series of Chromophores and Fluorophores Present in Elastin

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Purified elastin was hydrolysed with HCl and manipulated under conditions that minimized oxidation. Gel-permeation chromatography on polyacrylamide gel and ion-exchange chromatography on dextran cation-exchanger each resulted in the separation of a series of yellow fluorescent fractions. These hitherto unreported ampholytes have fluorescence spectra that approximate to that of the intact protein, and account for its characteristic optical properties. Since the coloured fluorophores are confined to enzyme-resistant regions of the protein molecule they appear to have important structural implications.

The peculiar elastomeric nature and fluorescence of elastin have occupied the attention of researchers for some years (Partridge, 1962). Although anatomists and histologists have traditionally referred to elastin as yellow connective tissue, recent theories on the detailed structure of this coloured protein leave the nature of both the chromophore and fluorophore unexplained. In fact, the optical properties of this material are seldom described when its structure is discussed (e.g. Gray *et al.*, 1973; Piez, 1968). The present results demonstrate that the characteristic colour and fluorescence are contained in a series of hitherto unreported ampholytes. These appear to be unique to elastin and important from the structural viewpoint.

Since the optical properties of elastin could not be accounted for by its amino acid composition, they were originally thought to be associated with the peculiar elastic properties and insolubility of the protein. Elastin contains very little cysteine, is insoluble in reagents that break disulphide bonds, and hence must be cross-linked by unusual bridges. In a series of classic experiments Partridge and co-workers attempted to isolate and characterize putative cross-links. Sequential digestion of elastin with a series of enzymes yielded enzyme-resistant residues (Partridge *et al.*, 1963). Size fractionation of these yielded 'H' peptides, so called because of their presumed cross-linking function between adjacent peptide chains. These residues retained the characteristic optical properties of the intact protein. They were bright yellow, fluoresced a brilliant blue-white under u.v. illumination, and had a pH-independent u.v. absorption. Subsequent acid hydrolysis and fractionation yielded the isomeric desmosines (Thomas *et al.*, 1963). These heterocyclic

amino acids were ideal candidates for cross-links because of their polyfunctional nature. Although they accounted for the u.v. absorption of the intact protein the desmosines were neither coloured nor fluorescent.

It was not clear how the desmosines could have contributed towards the colour of the protein or H peptides. Partridge *et al.* (1963) cautiously suggested that the desmosines might have been degradation products of the chromophore. Ayer (1964) considered colour to result from molecular interactions within the protein, and Podrazky (1968) used colour as a criterion to identify peptides containing desmosines. The problem of fluorescence did not appear to merit attention, and the origin of the chromophore and fluorophore originally present in the H-peptide residues remained obscure.

Recently, gel-permeation chromatography of the residues remaining after digestion of elastin with elastase and Pronase indicated that the chromophore, fluorophore and u.v.-absorbing residues were all confined to what may be presumed to be the cross-linked regions of the protein (Thornhill, 1972a), thus providing further association of the chromophore and fluorophore with cross-links. Conventional acid hydrolysis degraded both the chromophore and fluorophore but not the desmosines. Hydrolysis under reducing conditions minimized this degradation, and when freed in this manner the chromophores were shown to be distinct from the desmosines, since the latter were not adsorbed on charcoal (Thornhill, 1972a). Thus a tentative explanation of the fate of the chromophore and fluorophore originally present in the H peptides was that they were oxidized and/or adsorbed on Dowex-50 resin, leaving the desmosines to be recovered intact. New methods of amino acid

fractionation (Thornhill, 1972*b*, 1974) have permitted further examination of the residues obtained from acid hydrolysis of elastin.

Experimental

Elastin [4g of bovine ligamentum, previously purified by Thornhill's (1972*a*) method] was hydrolysed for 72h with 250ml of deoxygenated 6*M*-HCl that contained 30mg of stannous chloride. Hydrolysis was carried out under N₂ and access of O₂ prevented by a pyrogallol trap. Most of the HCl was evaporated on a rotary evaporator and the vacuum released with N₂. The hydrolysate was further evaporated at 4°C over NaOH under a vacuum that was released with N₂. The resulting golden-yellow syrup was divided into two portions. One portion (portion A) was titrated to pH2.5 with Dowex-1 (HCO₃⁻ form). The resin was rinsed, the washing combined with the original supernatant and the product was freeze-dried. The other portion (portion B) was not further treated.

The freeze-dried hydrolysate (portion A) was taken up in approx. 5ml of water and loaded on a column (5.0cm×50cm) of Bio-Gel P2 polyacrylamide gel (Bio-Rad Laboratories, Richmond, Calif., U.S.A.) that had been equilibrated with 0.5*M*-acetic acid that contained a trace of ascorbic acid. Elution was carried

out at room temperature and 10ml fractions were collected and monitored as described below.

The untreated portion (portion B) was diluted to approximately 10ml with water and loaded on a column (2.5cm×50cm) of sulphoethyl (SE)-Sephadex C-25 (Pharmacia Ltd., Montreal, Canada) that had been equilibrated with 0.5*M*-acetic acid. The column was eluted at 40°C with a linear NaCl gradient increasing from 0 to 2*M* in 0.5*M*-acetic acid. A trace of ascorbic acid was included to prevent oxidation. Fractions (10ml) were collected and monitored as described below.

In both separation procedures the E_{280} of each fraction was measured with a flow-cell (Instrument Specialties Co., Nebraska, U.S.A.). Fluorescence emission at wavelengths greater than 400 nm, with illumination at 340nm, was monitored with a filter instrument using a flow-cell as previously described (Thornhill, 1971) or in individual fractions. Fluorescence at 280nm activation/305nm emission was measured in the region where tyrosine was shown to have been eluted. Fluorescence spectra (uncorrected) of the major fluorescent peaks were determined with quartz cells, in acidic (HCl, pH1) and alkaline (NaOH, pH12) solution, on a grating instrument (Aminco-Bowman, American Instrument Co., Silver Spring, Md., U.S.A.). The instrument amplification (meter setting) was kept constant. Each fraction was examined by paper chromatography

Fig. 1. Elution behaviour of elastin hydrolysate on Bio-Gel P2 polyacrylamide gel

Elastin (2g) was hydrolysed with HCl under reducing conditions and the product titrated to pH2.5 with Dowex-1 (HCO₃⁻ form). The freeze-dried product was applied to a column (5cm×50cm) of Bio-Gel P2 polyacrylamide gel and 10ml fractions were eluted with 0.5*M*-acetic acid. Hatched areas indicate colour. Des, Desmosine isomers. ----, E_{280} (log scale); —, relative fluorescence emission at wavelengths greater than 400nm with illumination at 340nm (arbitrary units, linear scale); ●, relative fluorescence at 280nm activation/305nm emission (arbitrary units, linear scale). For details of peaks (I)–(IV) see the text and Fig. 3.

Fig. 2. Elution behaviour of elastin hydrolysate on SE-Sephadex C-25

Elastin (2g) was hydrolysed with HCl under reducing conditions and evaporated under a vacuum. The product was applied to a column (2.5 cm $\times$ 50 cm) of SE-Sephadex C-25 equilibrated with 0.5 M-acetic acid. Fractions (10 ml) were eluted by a 0–2 M linear gradient of NaCl in 0.5 M-acetic acid at 40°C. Hatched areas indicate colour. ----, E_{280} (log scale); —, relative fluorescence emission at wavelengths greater than 400 nm with illumination at 340 nm (arbitrary units, linear scale); ●, relative fluorescence at 280 nm activation/305 nm emission (arbitrary units, linear scale).

(Whatman no. 1), with development in butan-1-ol-acetic acid-water (12:3:5, by vol.). Amino acids were detected with the reagent of Moffat & Lytle (1959).

Results

Elution profiles of amino acids, colour, fluorescence and u.v. absorption are illustrated in Figs. 1 and 2. Fractionation on Bio-Gel P2 (Fig. 1) resulted in the resolution of three coloured zones that coincided with maximum fluorescence. In the desmosine region an unfamiliar amino acid was detected by paper chromatography. It migrated as a discrete zone, stained blue-violet with the ninhydrin spray reagent, and had an R_F value similar to that of tyrosine. Since it did not fluoresce it is not considered in this report. The desmosines were identified by their characteristic behaviour (Thornhill, 1972c). Before development, u.v. illumination of paper chromatograms indicated fluorescent fractions as brilliant blue-white discs, of similar appearance to native elastin. After development no discrete fluorescent zones were detectable, but faint fluorescence was streaked extensively over the paper.

Fractionation on SE-Sephadex likewise resulted in the separation of three yellow zones that coincided with regions of maximum fluorescence. Neither colour nor fluorescence was associated with the desmosine region. The behaviour of fluorescent fractions on paper chromatograms was similar to that of the fractions obtained by gel fractionation.

Discussion

The question whether the chromophore and fluorophore in elastin represents a single species was raised by Thornhill (1972a), since both appeared to have aromatic and ampholytic properties and had a similar locus in the protein molecule. The present results clearly indicate that a series of chromophores and fluorophores remain, apparently intact, in an elastin hydrolysate suitably prepared and fractionated under conditions that minimize oxidation and adsorption. Certain characteristics are evident when their elution behaviour from Bio-Gel P2 gel and SE-Sephadex are considered together. The sequence of elution of amino acids from Bio-Gel P2 gel has been investigated (Thornhill, 1972b), and acidic and aromatic amino acids are retarded and eluted in a similar region. Fluorescent peaks (III) and (IV) therefore appear to be retarded on Bio-Gel P2 gel as a result of their aromatic and/or acidic character. In contrast, peaks (I) and (II) are eluted in their respective positions from Bio-Gel P2 gel because they tend to be basic, or are relatively large. In acetic acid, the elution sequence of amino acids from SE-Sephadex is approximately comparable with that from Dowex-50 (Thornhill, 1974), except for aromatic species, and the desmosines which are eluted after arginine. Since no coloured or fluorescent fractions were eluted from SE-Sephadex before tyrosine, none of the peaks (I)–(IV) appear to be acidic ampholytes. Since all the fluorophores adsorb on to charcoal (Thornhill, 1972a) they appear to contain aromatic structures. Peaks (III) and (IV) therefore appear to be retarded

Fig. 3. Fluorescence spectra of fluorophores resolved on Bio-Gel P2 polyacrylamide gel

Activation and emission spectra (uncorrected) of fluorescent peaks (I)–(IV) obtained by separation of an elastin hydrolysate on Bio-Gel P2 polyacrylamide gel, as illustrated in Fig. 1. Fluorescence intensity is expressed in arbitrary units (meter reading) with a constant instrument amplification throughout. ----, Acid (HCl, pH 1); —, alkali (NaOH, pH 12).

on Bio-Gel P2 gel solely because of aromatic properties. Peaks (I) and (II) apparently are eluted early from Bio-Gel P2 gel because basic or size factors override aromatic properties. This is consistent with their elution position on SE-Sephadex. In neither fractionation procedure was colour separated from fluorescence. Except for the unfamiliar ninhydrin-reacting material in peak (I) from the Bio-Gel P2 gel column, which also appeared in the first fluorescent zone eluted from SE-Sephadex, no other unusual amino acids were detected.

The fluorescence spectra (Fig. 3) indicate that the non-basic aromatic fluorophores (peaks III and IV) exhibit brighter fluorescence in alkaline solution, with a bathochromic shift in the activation spectrum, distinguishing them from peaks (I) and (II).

These results clearly indicate that a group of coloured fluorescent ampholytes may be freed from elastin by hydrolysis and manipulation under reducing conditions. Two dissimilar methods of separation resolved several components and their fluorescence spectra closely approximate to that of the intact protein.

Although they are easily oxidized (Thornhill, 1972a), there is nothing to suggest that these residues

may be degraded to the desmosines. On the contrary, since they account for the hitherto unexplained characteristic colour and fluorescence of the native protein, they appear to be unique to elastin and are clearly not artifacts. Their entire location in enzyme-resistant regions of the protein molecule suggests that they are associated, in some fashion, with cross-links. There is a remarkable analogy to the chromophore and fluorophore that characterize another elastic protein, abductin. They too are confined to enzyme-resistant regions (Thornhill, 1971) and are not detectable by conventional methods of amino acid analysis.

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