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Amyloid A: Amphipathic Helixes and Lipid Binding†

J. P. Segrest,* H. J. Pownall,‡ R. L. Jackson,‡ G. G. Glenner, and P. S. Pollock

ABSTRACT: Polypeptide segments, composed of α helixes with specific surface topography termed amphipathic helixes, have been proposed as the basic lipid-associating domains of apolipoproteins from the plasma lipoproteins. A computer search for proteins having sequences that could form amphipathic helixes indicated that amyloid A, a pathologically occurring protein usually associated with "secondary" amyloidosis, also contained amphipathic helixes. In studies reported here, amyloid A is shown to associate spontaneously with phospholipid vesicles with the following results: (a) the formation of a protein–lipid complex isolated by equilibrium density gradient ultracentrifugation, (b) a 100% increase in α helicity as measured by circular dichroism, (c) a 9-nm shift in the fluorescence maximum due to the single tryptophan residue located in the amphipathic region, indicating the tryptophan is moving from a polar to a nonpolar environment, and (d) the formation of stacked disk-like protein–lipid complexes as visualized by negative stain electron microscopy. The temperature dependence of the circular dichroic spectrum of the amyloid A–phospholipid complex suggests that the complex is formed by insertion of protein between the fatty acyl chains of the lipid. These findings suggest that the amphipathic helix is an important structural unit in lipid-associating proteins and that this unit can be recognized on the basis of its amino acid sequence. In addition, these studies have implications for the origin and function of amyloid A protein.

An α helix with a specific surface topography consisting of opposing polar and nonpolar faces and a regular charge distribution (termed an amphipathic helix) has been proposed as a basic structural element of the lipid-associating domains of apolipoproteins in very low-density lipoproteins (VLDL)[1] and high density lipoproteins (HDL) (Segrest et al., 1974). Potential amphipathic helixes (to be referred to as amphipathic sequences) can be identified from amino acid sequence data. Using a computer program based on this model, amphipathic sequences have been identified from known amino acid sequence data (Segrest, J. P., and Feldmann, R. J., submitted for publication).

The computer search for amphipathic sequences identified two amphipathic sequences from the amino acid sequence of amyloid A. As one test for the amphipathic helix model for protein–lipid association, amyloid A was examined for lipid-associative properties.

Amyloid A (Glenner et al., 1973; Ein et al., 1972; Benditt

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[1] Abbreviations used are: VLDL, very low-density lipoprotein; HDL, high-density lipoprotein; DMPC, dimyristoylphosphatidylcholine; CD, circular dichroism; T_c, liquid crystalline phase transition temperature; EDTA, (ethylenedinitrilo)tetraacetic acid.

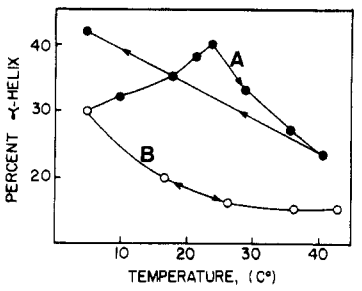

FIGURE 1: α-Helical content of amyloid A as a function of increasing and decreasing temperature. (A) Amyloid A alone. (B) Amyloid A–DMPC mixture (35:1) after mixing at 5 °C and recording the CD spectrum as a function of increasing and decreasing temperature.

et al., 1971; Husby and Natvig, 1974) is a pathologically occurring protein (or protein fragment) often associated with the clinical syndrome referred to as "secondary" amyloidosis; the amyloid protein appears following a chronic disease such as tuberculosis. The origin of this protein is unknown, whereas the protein deposited in body tissues in "primary" amyloidosis is derived from the light-chain portion of immunoglobulin (Glenner et al., 1973). Although there is no known similarity in the physiological roles of amyloid A and the plasma apolipoproteins, the results in the present report show that amyloid A spontaneously associates with phospholipid vesicles to yield a protein–lipid complex having many of the structural properties observed in complexes of plasma apolipoprotein and lipid.

Materials and Methods

Amyloid A. Amyloid A was isolated by gel filtration after guanidine-HCl denaturation of splenic fibril concentrates from a patient with systemic amyloidosis and rheumatoid arthritis (Ein et al., 1972). The isolated protein contained less than 0.5% phospholipid as determined by phosphorus analysis (Bartlett, 1959).

The amino acid sequence of amyloid A is shown in Figure 5. The particular form of the protein used in the studies reported here is 45 amino acid residues in length (Ein et al., 1972) and will be designated amyloid A-1. A second form of amyloid A, 76 residues in length, to be designated A-2, has also been reported (Benditt et al., 1971). Amyloid A-2 is identical to the shorter version with the addition of 31 COOH-terminal residues. The 45 residue fragment, amyloid A-1, contains one amphipathic sequence of 26 residues, identified by computer search, located at the NH_2-terminal end (residues enclosed in circles in Figure 5 lower). This amphipathic segment represents 58% of the molecule. Amyloid A-2 contains one additional amphipathic sequence (Segrest and Feldmann, submitted for publication).

Phospholipids. Egg lecithin was obtained from Avanti Biochemicals, Inc., and was shown to be chromatographically pure (Singleton et al., 1965; Rouser et al., 1963; Dittmer and Lester, 1964). ^{14}C-Labeled dimyristoylphosphatidylcholine (DMPC), labeled at the C-1 position of both acyl chains, was custom synthesized by Applied Science. The [^{14}C]DMPC was chemically and radiochemically pure by thin-layer chromatography (Singleton et al., 1965; Rouser et al., 1963; Dittmer and Lester, 1964) and gas chromatography of its methyl esters (Morrison and Smith, 1964).

Liposomes. Single bilayer vesicles (liposomes) were prepared by ultrasonic irradiation of the phospholipid (10–30 mg/ml) (Biosonik IV with a microtip probe) for 30 min at 5–15 °C for egg lecithin and 23–30 °C for DMPC in a buffered (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 1 mM NaN_3) aqueous solution (Prestegard and Fellmeth, 1974). Single bilayer vesicles were isolated by gel filtration on Sepharose 4B (Huang, 1969) or by centrifugation at 20 000*g* for 20 min (Papahadjopoulos et al., 1974). All experiments were performed within 24 h of the preparation of the vesicles.

Circular Dichroic Spectra. The circular dichroic (CD) spectra were obtained as a function of temperature as previously described (Pownall et al., 1974). Amyloid A (0.42 mg/ml) and DMPC (1.9 mg/ml) were cooled for 30 min at 4 °C and combined in an 0.5-mm cell that was quickly transferred to the precooled (5 °C) cell compartment of a Cary 61 spectropolarimeter. The CD spectra were recorded with increasing temperature with a 30-min thermal equilibration of the sample at each temperature before recording the spectrum. The sample was heated to a maximum temperature of 41 °C, after which an additional measurement was recorded at 5 °C. An identical procedure was followed in which the addition of DMPC to the sample was omitted. The α-helical content of the protein was estimated according to a modification (Morrisett et al., 1973) of the procedure of Greenfield and Fasman (1969).

Linear Density Gradient Ultracentrifugation. The entire sample of DMPC and amyloid A used in the CD experiment was distributed in a linear density gradient of KBr as previously described (Morrisett et al., 1973). The sample was centrifuged for 96 h at 45 000*g* in a Beckman SW 50.1 rotor at 5 °C. A small sample of the protein, approximately 0.08 mg, was spun simultaneously. At the end of the centrifugation period, each sample was fractionated and analyzed for density (by refractive index), protein (A_{280}) and DMPC (by liquid scintillation counting of a 100-μl aliquot of each fraction). Amyloid A concentration was determined by amino acid analyses using standard methods.

Fluorescence Spectroscopy. Fluorescence spectra were recorded on an Aminco-Bowman Spectrofluorimeter with excitation at 280 nm as previously described (Morrisett et al., 1973).

Negative-Stain Electron Microscopy. Negative-stain preparations were made of amyloid A and egg lecithin complexes (2–3 mg/ml at a molar ratio of 1:50) with 0.1% phosphotungstic acid at pH 7.0 by the procedure of Haschemeyer and Myers (1972). The preparations were examined and photographed on a Philips 200 electron microscope.

Results

CD Spectra. Amyloid A contains 30% α-helical structure at 5 °C and this decreases to about 15% when the temperature is raised to 43 °C (Figure 1B). The changes in α-helical content were reversible between these two temperatures. After mixing the DMPC and amyloid A at 5 °C, the CD spectrum of the mixture was identical with that of the protein alone (Figure 1A); i.e., the protein contained 30% α-helical structure. As the temperature increased, the α helicity of the protein increased to a maximum at 24 °C, after which the helicity decreased. Cooling of the lipid–protein mixture through the same temperature range was irreversible and the final α helicity of the mixture at 5 °C after one heating and cooling cycle was 42%; i.e., a 40% increase in the α helicity of the protein resulted relative to the protein without DMPC.

Isolation of a Lipid–Protein Complex. Immediately after the measurements of the CD spectra of the protein and lipid–protein solutions, the lipid–protein complex was isolated in a linear density gradient. As shown in Figure 2A, centrifugation

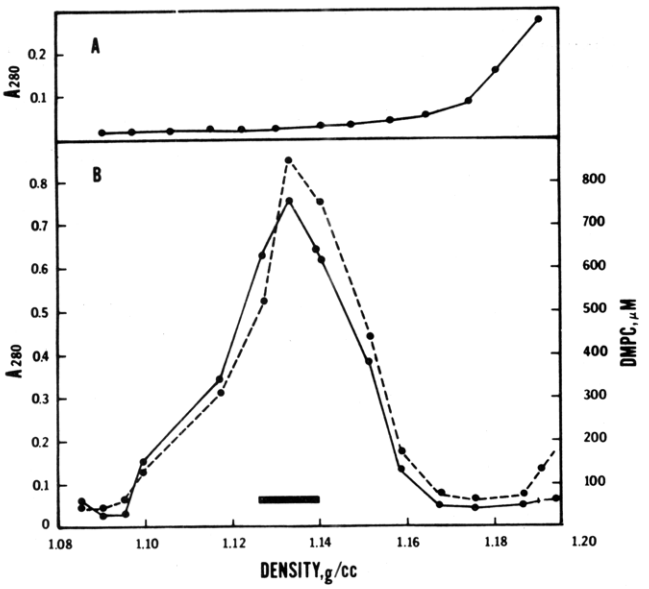

FIGURE 2: Composition vs. density profile of amyloid A. (A) Protein alone. (B) Amyloid A–DMPC mixture after centrifugation to equilibrium. The density of DMPC is ≃1.06 (Kruski and Scanu, 1974). The samples were the same ones used to obtain the data of Figure 1.

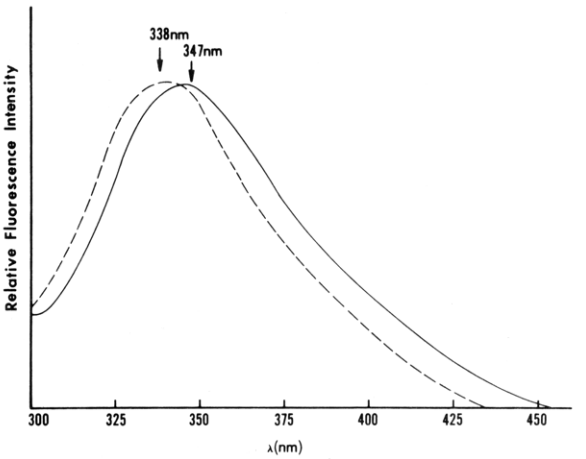

FIGURE 3: Fluorescence spectra of amyloid A alone (maximum = 347 nm) and the amyloid A–DMPC (1:125) complex isolated by density gradient ultracentrifugation (maximum = 338 nm). The fluorescence maximum of amyloid A in 6 M guanidine hydrochloride is at 353 nm.

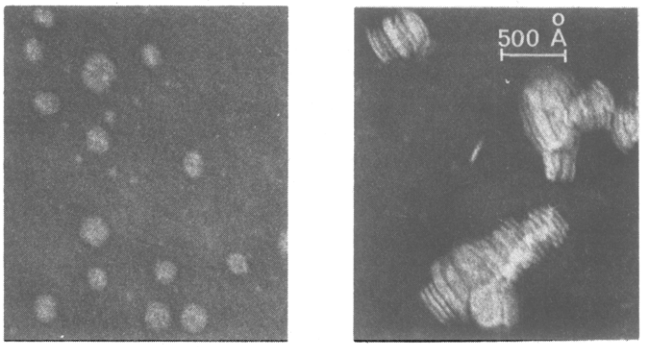

FIGURE 4: Negative stain electron micrograph of amyloid A–egg lecithin complex (1:35). (A) Egg lecithin alone. (B) Protein–lipid complex.

of the protein alone resulted in the sedimentation of the protein. Independent experiments (Kruski and Scanu, 1974) have shown that DMPC alone sediments at $\rho \simeq 1.06$. The mixture of amyloid A and DMPC, however, appears (Figure 2B) at a density between those of each of the components, as one would expect for a stable lipid–protein complex (Morrisett et al., 1973; Pownall et al., 1974; Kruski and Scanu, 1974; Jackson et al., 1974). Determination of the stoichiometry of the complex showed a DMPC–amyloid A stoichiometry of 125:1; a portion of this complex was used in the fluorescence studies.

Fluorescence Spectra. The intrinsic fluorescence of amyloid A in the absence of DMPC exhibited a spectral maximum at 347 nm (Figure 3). The fluorescence maximum of the DMPC–amyloid A complex, after dialysis against 100 mM NaCl, 1 mM sodium azide, 1 mM EDTA, and 10 mM Tris, pH 9.0, to remove the KBr, is shown in Figure 3. The spectral maximum of the fluorescence of the DMPC–amyloid A complex appearing at 338 nm represents a total wavelength shift of 9 nm from amyloid A alone.

Negative-Stain Electron Microscopy. As seen from Figure 4, association of amyloid A with single bilayer egg lecithin liposomes converted the spherical vesicles to flattened disk-shaped structures with thicknesses of approximately 50 Å, equivalent to the thickness of a single phospholipid bilayer. These changes are identical to those seen following association of plasma apolipoprotein with liposomes (Hoff et al., 1973). At a protein–DMPC stoichiometry of 1:125 each vesicle corresponds to approximately 30 amyloid A molecules, assuming a vesicular aggregation number of 2500 (Huang, 1969).

Discussion

Interpretation of Results. The spontaneous association of amyloid A-1 with phospholipid vesicles is characteristic for lipid-binding proteins, such as those of the plasma lipoproteins. Ordinary globular proteins will not spontaneously associate with lipid to produce the CD, fluorescence, and electron micrographic changes described here for amyloid A-1 (Morrisett et al., 1973).

The CD studies suggest that amyloid A does not interact with DMPC at 5 °C. As the temperature was increased there was a gradual increase in protein–lipid association (manifested by an increase in α-helix content) with an increase in association near the gel to liquid crystalline phase transition temperature (T_c = 23 °C) of DMPC. In contrast to amyloid A, apolipoprotein C-III displays a much sharper increase in α-helix content in the region of the T_c (Pownall et al., 1974). One possible explanation for this difference is that amyloid A–DMPC association is more exothermic than that of C-III–DMPC association resulting in a cooperative focal melting of the DMPC gel phase near the association sites. The protein, free or complexed with DMPC, appears to have a fairly loose structure, since an increase in temperature produced a significant decrease in α helicity.

The amyloid A–DMPC complex showed two kinds of irreversibility upon cooling through the temperature range 43–5 °C (Figure 1). First, the protein apparently remained associated with the lipid even below the T_c. Second, the helical content did not follow the previous temperature dependent path in reverse. At least two possibilities can explain the second finding. Either there is dissociation of protein with increased temperature in an irreversible fashion or the relatively high α helicity seen at the T_c is a metastable phenomenon that does not recur upon heating and cooling. The latter seems the more likely possibility.

Complex formation between amyloid A and DMPC resulted in an increase in α helicity of the protein from 30 to more than 42% (see below). Further, the association is kinetically inhib-

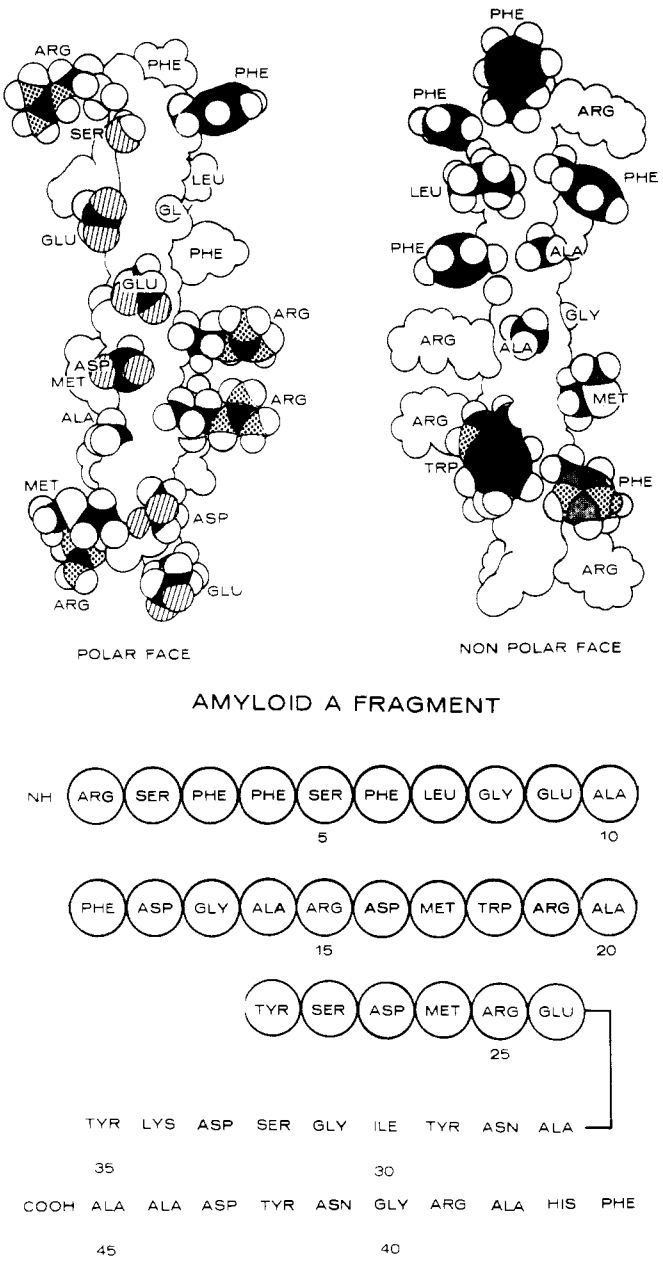

FIGURE 5: Amphipathic helical segment from amyloid A. (Lower) Amino acid sequence of amyloid A. Amphipathic segment is indicated by circles. Note tryptophan at residue 18. (upper) CPK space-filling model of amphipathic segment (residues 1-26) built as an α helix showing polar and nonpolar "faces".

ited below the T_c (in the gel phase) and is much more rapid above the T_c (in the liquid crystalline phase). This suggests that amyloid A-DMPC association involves insertion of protein between the fatty acyl chains of the lipid.

The linear density gradient showed that amyloid A and DMPC associate to form a stable protein-lipid complex with a stoichiometry of 1:125. There was a small amount of free protein found in the most dense fractions of the density gradient so that the 42% helix calculated from the CD spectrum represents a lower limit.

The interpretation of the difference in the intrinsic fluorescence spectrum of amyloid A, free and complexed to DMPC, is that the single tryptophan residue in the protein (see Figure 5) is in a more hydrophobic environment following lipid association. This change could represent either burial within the protein following some conformational change (or entrapment between subunits) or penetration of the tryptophan residue into the hydrocarbon milieu of the fatty acyl chains of DMPC.

Amphipathic Helix Model. The amphipathic sequence contained in amyloid A-1 (and in A-2) is shown as an α helix in Figure 5 (upper) constructed from CPK space-filling models. A polar and an opposing nonpolar "face" are apparent, as first identified in the amino acid sequences of plasma apolipoproteins A-I, A-II and C-II and C-III (Segrest et al., 1974).

It is informative to compare the predictions of the amphipathic helix model with the results of the experiments reported here. The model predicts that the nonpolar face would insert between the fatty acyl chains of hydrated DMPC producing an increase in α helicity with lipid association (Segrest et al., 1974). The model predicts that the tryptophan residue at position 18 (Figure 5) would be buried in the fatty acyl chains of hydrated DMPC. These predictions are supported by the results of the CD and fluorescence experiments. Finally, the flattened disks seen when the amyloid A-1-phospholipid complex is examined by negative stain electron microscopy are predicted by the amphipathic helix model on the basis of expected geometrical changes resulting from an amphipathic helix-phospholipid complex (Segrest, J. P., *Chem. Phys. Lipids* (in press)).

While the findings reported here do support the amphipathic helix model as an important structural unit in lipid associating proteins, other possibilities cannot be excluded. For example, there is a small but finite possibility that the simultaneous presence of amphipathic sequences and lipid-associating properties in amyloid A is totally fortuitous. Further, we have not shown that the portions of amyloid A involved in lipid binding and the formation of α-helical structure are the same and identical to the postulated amphipathic sequence shown in Figure 5.

Origin and Function of Amyloid A. Given that amyloid A-1 associates with phospholipid vesicles, what might be the origin and function of this protein fragment? It seems likely that lipid-binding is one property of the original protein. However, it should be noted that amyloid A-1 represents a denatured fragment of a presumably longer polypeptide; without isolation of the whole protein any discussion of its function must remain conjectural.

With this qualification in mind, since amyloid has been detected in plasma (Husby and Natvig, 1974), amyloid A-1 may represent a fragment of some previously unrecognized plasma apolipoprotein. However, this possibility does not explain the association of secondary amyloidosis with chronic stimulation of the immune system.

It seems more likely that amyloid A-1 is a fragment of a membrane-binding protein released into the sera (either primarily or secondarily) upon antigenic stimulation. For example, amyloid A-1 might be a fragment of an attachment protein for membrane-associated immunoglobulins or a member of the complement system. Confirmation of this or any other possibility for the origin and function of amyloid A awaits further studies directed toward this protein.

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The Identity of a Cyanogen Bromide Fragment of Bovine Dentin Collagen Containing the Site of an Intermolecular Cross-Link†

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ABSTRACT: A peptide fraction isolated from a cyanogen bromide digest of bovine dentin collagen had a molecular weight of 46 000. Its size and amino acid composition indicated that it could not consist of peptides derived from the cleavage of a single α chain. On reduction with tritiated sodium borohydride, radioactivity was incorporated primarily into 5,5′-dihydroxylysinonorleucine without degradation at the peptide backbone. Periodate cleavage of the reduced or nonreduced peptide fraction generated one fragment of molecular weight 28 000 and one of 18 000 completely accounting for the size of the parent peptide. On amino acid analysis the constituent single-chain peptides were determined to be α2CB4 and α1CB6. Both peptides isolated after periodate oxidation of the tritiated borohydride reduced cross-link peptide were found to contain [^{3}H]hydroxynorvaline. These data show that some hydroxylysine of α2CB4, a helical region peptide, was present in aldehyde form and could act as the aldehyde donor in cross-link, Schiff's base formation. The only cross-linkage of this α2CB4 acting as an aldehyde donor peptide to α1CB6 would be a helical region to helical region bond, perhaps accounting for the unusual stability and low solubility of dentin collagen.

Bone and dentin collagens are stabilized by interchain cross-links of the Schiff base type, involving lysine or hydroxylysine and hydroxyallysine (Mechanic et al., 1971; Davis and Bailey, 1971), the aldehyde formed by oxidative deamination at the ϵ-amino group of hydroxylysine. The Schiff base may then be stabilized by reduction in vivo (Mechanic et al., 1971), by rearrangement to the corresponding ketimine (Miller and Robertson, 1973; Eyre and Glimcher, 1973a; Mechanic et al., 1974), or possibly by condensation with sterically available lysine or hydroxylysine residues on adjacent α chains (Davis et al., 1975).

Little is presently known about the number or location of such cross-links in insoluble collagens, although several authors have stressed the importance of lysine and hydroxylysine residues which may be oxidized to the corresponding aldehydes (Kang et al., 1967; Rauterberg et al., 1972), and which are present in the nonhelical terminal extensions of the α chains. Kang (1972) demonstrated the presence, in sodium borohydride reduced rat tail tendon collagen, of a cross-link peptide which involved the amino terminus of one α1 chain and the carboxy terminus of an adjacent α1 chain. This was purified and determined to be the cross-linked CNBr[1] peptide—(α1CB1 × α1CB6). Dixit and Bensusan (1973) isolated the analogous peptide from a CNBr digest of reduced insoluble bovine corium collagen, together with two others, both involving the carboxy-terminal CNBr peptide of the α1 (I) chain—α1CB6. Volpin and Veis (1973) and Zimmerman et al. (1973) found the yield of this latter peptide to be signifi-

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[1] Abbreviations used: CNBr, cyanogen bromide; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; DHLNL, dihydroxylysinonorleucine; HLNL, hydroxylysinonorleucine.