Identification and Properties of the Covalently Bound Flavin of β-Cyclopiazonate Oxidocyclase†

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ABSTRACT: β-Cyclopiazonate oxidocyclase from *Penicillium cyclopium* has been previously shown to contain flavin dinucleotide in covalent linkage to the protein. In the present study, a pure flavin mononucleotide peptide was isolated from the enzyme by tryptic–chymotryptic digestion, chromatography on Florisil and on diethylaminoethylcellulose, and hydrolysis with nucleotide pyrophosphatase. The flavin peptide contains 9 amino acids, including histidine in linkage to the flavin, and Asx as the N-terminal residue. The fluorescence of the flavin in the FMN peptide is profoundly quenched even at pH 3.2, where protonation of the imidazole prevents quenching of the flavin fluorescence by histidine. This quenching appears to be due to interaction of the flavin with a tryptophan residue, as the quenching is abolished by oxidation of the tryptophan with performic acid. Similarly, the fluorescence of the tryptophan in the peptide is quenched, presumably by the flavin. The flavin of β-cyclopiazonate oxidocyclase is attached, by way of the 8α-methylene group, to the imidazole ring of a histidine. The aminoacylflavin isolated from the enzyme is identical in the pK_a of its imidazole group, in reduction by $NaBH_4$, and in other properties with synthetic 8α-(N^1-histidyl)riboflavin. The pK_a of the histidylriboflavin component of the oxidocyclase is 5.2 before and 5.0 after acid modification of the ribityl chain, as is found in the synthetic derivative. It is concluded that the enzyme contains the N^1 isomer of histidylriboflavin and that acid hydrolysis of flavin peptides isolated from the oxidocyclase, while liberating histidylriboflavin, also causes acid modification of the ribityl chain of the flavin moiety.

β-Cyclopiazonate oxidocyclase, an enzyme from *Pencillium cyclopium,* catalyzes the dehydrogenation and cyclization of β-cyclopiazonic acid to α-cyclopiazonic acid. It has been shown that the homogeneous enzyme contains FAD[1] in covalent linkage to the protein (Schabort and Potgieter, 1971). Since the properties of the partially purified flavin peptide released by proteolytic digestion suggested that its aminoacylflavin moiety might differ from covalently bound flavins previously reported, a collaboration was established between the laboratories in San Francisco and South Africa in order to define the structure of the flavin. In a brief communication (Kenney et al., 1974c), we presented preliminary evidence indicating that the flavin component of β-cyclopiazonate oxidocyclase is linked to the imidazole ring of histidine, as in the case of succinate dehydrogenase (Walker et al., 1972). The histidylflavin of β-cyclopiazonate oxidocyclase differs, however, from 8α-(N^3-histidyl)riboflavin or its acid-modified form in the pK_a value of the imidazole ring nitrogen, as judged by pH-dependent fluorescence-quenching profiles.

A synthetic histidylriboflavin with properties indistinguishable from the aminoacylflavin moiety of β-cyclopiazonate oxidocyclase was subsequently obtained as a by-product of the chemical synthesis of 8α-(N^3-histidyl)riboflavin (Singer and Edmondson, 1974; Singer et al., 1976). This compound has recently been unambiguously characterized as 8α-(N^1-histidyl)riboflavin. The compound previously believed to have this structure (Walker et al., 1972) has been demonstrated to be an N^3 isomer of histidylriboflavin which is modified in the ribityl chain (Edmondson et al., 1976).

This paper presents detailed evidence that the flavin component of β-cyclopiazonate oxidocyclase is 8α-(N^1-His)FAD. The unusual property of flavin peptides derived from the enzyme of intensively quenched flavin fluorescence at pH 3.2 (where the nitrogen of imidazole is fully protonated and thus incapable of quenching the fluorescence of riboflavin) is shown to be due to interaction with a tryptophan in the peptide chain. This interaction is also shown by the quenching of tryptophan fluorescence by the flavin in the peptide.

Experimental Procedures

Materials. The following materials were obtained from the companies listed: DEAE-cellulose (DE-52), W. & R. Balston, Ltd., England; Florisil (F100, 60–100 mesh), Supelco, Inc.; trypsin and α-chymotrypsin, Worthington Biochemical Corp.; and nucleotide pyrophosphatase (type II from *Crotalus adamanteus*), Sigma. 8α-(N^1-histidyl)riboflavin and 8α-(N^3-histidyl)riboflavin were synthesized and purified as previously described (Walker et al., 1972; Edmondson et al., 1976). The tryptic peptide of succinate dehydrogenase was obtained by published methods (Kenney et al., 1972).

Purification of the Flavin Peptide. β-Cyclopiazonate oxidocyclase, purified as in earlier studies (Schabort et al., 1971), was treated with 5% (w/v) trichloroacetic acid at 0 °C and the precipitated protein was collected by centrifugation for 10 min at 29 000*g*. The precipitate was suspended in 0.1 M Tris to a protein concentration of 30 mg/ml; the pH was adjusted to 8.0, and the precipitate was digested in the dark with trypsin and chymotrypsin (0.1 mg each per mg of protein) for 4 h at 38 °C. After cooling to 0 °C, 0.1 volume of 55% (w/v) trichloroacetic acid was added and the flavin peptides were separated from

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[1] Abbreviations used are: FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; DEAE, diethylaminoethyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

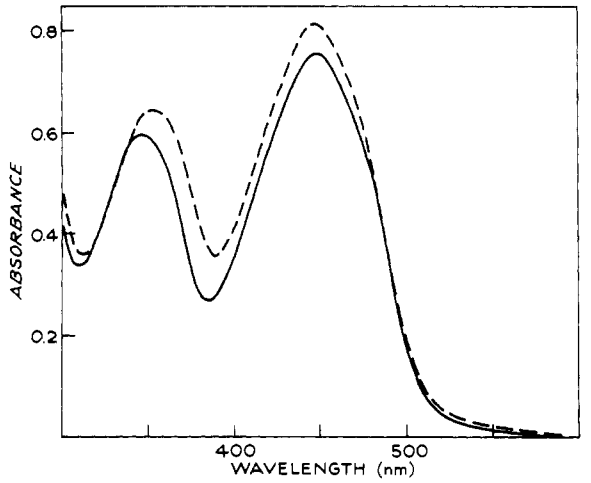

FIGURE 1: Absorption spectra of flavin peptide of β-cyclopiazonate oxidocyclase. FMN level: in 0.06 M ammonium bicarbonate, pH 7.2 (- - -); in 0.06 M acetic acid, pH 3.2 (—).

acid-insoluble material by centrifugation, as described above. The precipitate was washed once with 5% (w/v) trichloroacetic acid. The combined supernatants (containing 800 nmol of flavin) were applied at 4 °C to a 2.0 × 12 cm column of Florisil equilibrated with 5% (v/v) acetic acid. The column was washed with 5% acetic acid, until the absorption at 280 nm was <0.03, and then washed with water, until the pH of the eluent reached 5.0. The flavin peptides were then eluted with 5% (v/v) pyridine and the solvent was removed by lyophilization.

The flavin, dissolved in a minimum volume of pyridine, was applied to a 0.9 × 15 cm column of DEAE-cellulose, equilibrated with a solution containing 0.05 M each of pyridine and acetic acid, pH 5.0. The column was washed in succession with 15 ml each of 2% pyridine, H_2O, and then 0.01 M pyridinium acetate. The flavin peptide was eluted with a linear gradient of pyridinium acetate (0.05–0.50 M). The fractions containing the flavin were pooled and taken to dryness.

The flavin peptide (1 mM) was incubated with nucleotide pyrophosphatase (0.25 mg/ml final concentration) in 0.05 M Tris-HCl, pH 7.4, containing 10 mM $MgCl_2$, for 2.5 h at 38 °C and then taken to dryness. It was then dissolved in a minimum volume of pyridine and subjected to chromatography on DEAE-cellulose, exactly as described above.

Cleavage of the Flavin–Peptide Linkage. The flavin moiety was released from the flavin peptides by methylation and reductive cleavage as described previously (Walker et al., 1972; Edmondson et al., 1976). Under the conditions used, no incorporation of $^{14}CH_3I$ was found in the flavin moiety of synthetic 8α-(N^1-histidyl)riboflavin. After solvent removal, the residue, dissolved in a minimum volume of 8% formic acid (v/v), was applied to Whatman no. 1 paper, and subjected to high-voltage electrophoresis at pH 1.6. The fluorescent component, with a mobility corresponding to that of FMN, was eluted with water and taken to dryness. One nanomole of this material was incubated in 0.2 ml of 50 mM acetic acid in vacuo at 98 °C for 21.5 h. An aliquot was subjected to thin-layer chromatography on cellulose plates (MN 300, Macherey-Nagel and Co.) in 1-butanol–acetic acid–water, 12:3:5 (v/v/v), in order to identify the flavin component.

Other Methods. Flavin concentrations were determined from spectra obtained with a Cary 14 recording spectrophotometer, assuming molar extinction coeffients at 450 nm of 11 300 for dinucleotides and 12 000 for mononucleotides. Corrected fluorescence spectra were measured on a Hitachi Perkin-Elmer Model MPF-3 spectrophotometer. Circular dichroic spectra were measured at 22 °C using a Jasco UV 5 instrument with a Sproul Scientific SS10 modification.

Amino acid content was determined on a Beckman 121 C amino acid analyzer after hydrolysis in vacuo in 6 N HCl, 107 °C, 18 h. Tryptophan was determined by the method of Spies and Chambers (1949). The aminoacylflavin was obtained by incubation of the flavin peptide (0.4 mM) in vacuo in 6 N HCl for 17 h at 95 °C and subsequent purification by thin-layer chromatography (cellulose support; 1-butanol–acetic acid–water, 4:1:5 (v/v/v), upper phase).

Histidine was liberated from the histidylflavin by incubation at 125 °C under the conditions above.

Ninhydrin spray (3% (w/v) in 90% aqueous 1-butanol containing 5% acetic acid) was used to detect α-amino groups and the Pauly reaction (Pauly, 1915) was used for the detection of histidine. Oxidations with performic acid were carried out as previously described (Walker et al., 1971). High-voltage electrophoresis was performed in a Misco apparatus for 1 h at 40 V/cm at pH 6.25 (0.5% (v/v) acetic acid–10% (v/v) pyridine) or at pH 1.6 (8% (v/v) formic acid).

Results and Discussion

Evidence for 8α Linkage to Flavin. A characteristic property of peptides containing covalently linked flavins is the hypsochromic shift of the second absorption maximum relative to the 372-nm maximum of FMN or riboflavin. This property has become a routine criterion for the assignment of the site of peptide substitution to the 8α position of the flavin moiety, since it is characteristic of all naturally occurring and chemically synthesized 8α-substituted flavins (Singer and Kenney, 1974).

The tryptic–chymotryptic flavin peptide of β-cyclopiazonate oxidocyclase has already been shown to exhibit this hypsochromic shift of the second maximum of the corrected fluorescence excitation spectrum at pH 3.4 (Kenney et al., 1974a). This property is also found in the absorption spectrum of this peptide (Figure 1). Thus, the absorption maxima are 354 and 447 nm at pH 7.2 and 346 and 448 nm at pH 3.2. Final proof of 8α substitution came from acid hydrolysis of the flavin peptide to histidylflavin and the demonstration that this was identical with 8α-(N^1-histidyl)riboflavin (cf. below).

Amino Acid Composition of Tryptic–Chymotryptic Flavin Peptide. The fact that histidine is the amino acid substituent on the 8α position of the flavin, and that the linkage involves an imidazole nitrogen comes from the following evidence. The aminoacylflavin, derived by acid hydrolysis (cf. Experimental Procedures) of the tryptic–chymotryptic flavin peptide, was found to be ninhydrin positive but to give a negative Pauly test for free histidine. After drastic acid hydrolysis (125 °C, 6 N HCl, 16 h), however, an amino acid was liberated which cochromatographed with histidine in 1-butanol–acetic acid–water, 4:1:5: (v/v/v), upper phase, on cellulose plates and gave a positive Pauly reaction.

That one of the imidazole nitrogens of histidine is involved in the linkage to the flavin is evident from the pK_a of fluorescence quenching (Figure 2A), which has been found to be indicative of the presence of a tertiary nitrogen at the 8α position of the flavin (Salach et al., 1972; Kenney and Walker, 1972). Subjecting data of Figure 2A to treatment via the Henderson–Hasselbalch equation results in a pK_a of fluorescence quenching of 5.85 for the tryptic–chymotryptic flavin peptide after performic acid oxidation and of 5.05 for the aminoacyl flavin obtained from the flavin peptide (Figure 2B). It should be mentioned at this time that the fluorescence of the peptide

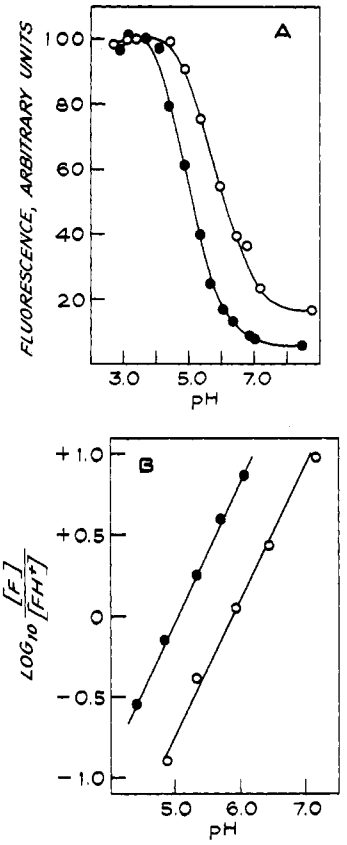

FIGURE 2: pH dependence of fluorescence of flavin peptide and aminoacylflavin. (A) Fluorescence of flavin peptide (○-○) was determined after performic acid oxidation; (●-●) aminoacylflavin from β-cyclopiazonate oxidocyclase. (B) Data of Figure 3A substituted into Henderson-Hasselbalch equation to determine pK_a of fluorescence quenching. FH^+ is protonated form of histidylflavin and F its conjugate base.

is extensively quenched (ca. 20% of that expected from the absorption spectrum) and that the fluorescence quenching properties become readily apparent only after oxidation of the peptide with performic acid (Kenney et al., 1974c).

Table I presents the amino acid composition of the flavin peptide isolated from β-cyclopiazonate oxidocyclase. Attention is called to the presence of tryptophan. As shown below, the intense quenching of the fluorescence at pH 3.2 results from the interaction of this residue with the flavin.

The amino terminal residue of the tryptic-chymotryptic flavin peptide was found to be Asx by the subtractive Edman method (Hirs et al., 1960) with Gly as the next residue.

Identity of the Histidylflavin of β-Cyclopiazonate Oxidocyclase with 8α-(N^1-His)FAD. Having determined that the 8α substituent of the flavin of β-cyclopiazonate oxidocyclase is histidine, it remained only to determine whether the linkage was to the N^1 or to the N^3 position of the imidazole ring. The properties of 8α-(N^1-histidyl)- and 8α-(N^3-histidyl)riboflavin and their respective acid-modified forms have previously been documented (Walker et al., 1972; Edmondson et al., 1976). As shown in Table II, the properties of the histidylflavin from β-cyclopiazonate oxidocyclase are identical with those of 8α-(N^1-histidyl)riboflavin in pK_a of fluorescence quenching, electrophoretic mobility, and in reduction by BH_4^- but differ significantly from 8α-(N^3-histidyl)riboflavin in these same properties. Acid treatment of the flavin peptide results in an acid-modified histidylflavin, as well as the parent 8α-(N^1-histidyl)riboflavin (Table II). This property is also observed with synthetic 8α-(N^1-histidyl)riboflavin (Edmondson et al., 1976). The structure of the acid-modified form has recently been found to be 8α-(N^1-histidyl)-2′,5′-anhydroriboflavin.[2] Proof that unmodified riboflavin (rather than its acid derivative) is present in the enzyme itself was obtained by methylating the FMN peptide with CH_3I, subjecting the product to reductive Zn cleavage and dephosphorylation, and demonstrating that in thin-layer chromatography the cleavage product is riboflavin itself, not its acid-modification product. It may be concluded from the foregoing that the structure of the covalently bound flavin of β-cyclopiazonate oxidocyclase is 8α-(N^1-His)FAD (Figure 3).

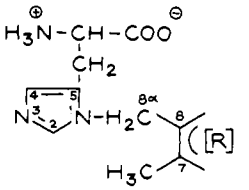

FIGURE 3: Structure of covalently bound flavin of β-cyclopiazonate oxidocyclase. R is rest of flavin.

TABLE I: Amino Acid Composition of Tryptic-Chymotryptic Flavin Peptide of β-Cyclopiazonate Oxidocyclase.

Amino Acid	Mol Found/ Mol of Flavin	Nearest Integer Amino Acid:Flavin
His	0.47	1[a]
Asx	1.91	2
Thr	1.89	2
Pro	1.07	1
Gly	1.03	1
Val	1.28	1
Trp	0.87	1

[a] As histidylflavin.

Evidence for Interaction between Tryptophan and Flavin. As noted above and documented in our preliminary note (Kenney et al., 1974c), the fluorescence of the FMN peptide from β-cyclopiazonate oxidocyclase is extensively quenched even at acid pH values, where quenching by the imidazole is precluded but this quenching is abolished on oxidation with cold performic acid. In contrast, after degradation to the histidylriboflavin level, the quenching is not seen; the fluorescence is not influenced by performic acid and equals that of FMN (Kenney et al., 1974c). It was concluded at the time that fluorescence quenching at pH 3.2 in the flavin peptide might be due to an interaction with an aromatic residue, as in *Chromatium* cytochrome c_{552} (Kenney et al., 1974a).

The demonstration (Table I) that the FMN peptide from β-cyclopiazonate oxidocyclase contains tryptophan is in accord with this prediction. The fact that either acid hydrolysis of the peptide (Figure 2A), which removes the tryptophan, or oxidation with cold performic acid, which destroys it, results in a great enhancement of fluorescence (Kenney et al., 1974c) shows that the quenching of flavin fluorescence in the peptide is due to interaction with tryptophan. Such interaction is also evident when the fluorescence of the tryptophan residue is examined (Table III). While tryptophan fluorescence is not markedly affected by the presence of an equimolar quantity of 8α-(N^1-histidyl)riboflavin in the solution, when both residues are present in the same peptide, a strong quenching be-

[2] D. E. Edmondson, manuscript in preparation.

TABLE II: Comparison of Properties of Histidylflavin from β-Cyclopiazonate Oxidocyclase with Those of Synthetic 8α-Histidylflavin Derivatives.

Property	Histidylflavin of β-Cyclopiazonate Oxidocyclase	8α-(N^1-Histidyl)riboflavin		8α-(N^3-Histidyl)riboflavin	
		Unmodified	Acid-Modified	Unmodified	Acid-Modified
pK_a of imidazole	5.05	5.2	5.0	4.7	4.5
Electrophoretic mobility at pH 6.25 (FMN = +1)	−0.34, −0.41[a]	−0.40	−0.34	−0.20	−0.19
Flavin product after methylation and reductive zinc cleavage	Riboflavin[b]	Riboflavin	Acid-modified riboflavin	Riboflavin	Acid-modified riboflavin
Reduced by BH_4^-	Yes[b]	Yes	Yes	No	No

[a] The yield of the faster migrating component was ca. 10% that of the slower component. [b] Determined on tryptic-chymotryptic flavin peptide.

TABLE III: Fluorescence Yield of Tryptophanyl Residues in 8α-Histidylflavin Peptides.[a]

Sample	Fluorescence	
	pH 3.3	pH 7.0
Tryptophan	100.0	107.8
Tryptophan + equimolar 8α-(N^1-Histidyl)riboflavin	95.0	100.9
β-Cyclopiazonate oxidocyclase flavin peptide	7.7	5.8
Tryptic succinate dehydrogenase flavin peptide	20.5	24.9

[a] Tryptophanyl fluorescence was measured at the corrected emission maximum which was 355 nm for free tryptophan, 375 nm for the β-cyclopiazonate oxidocyclase flavin peptide, and 360 nm for the tryptic succinate dehydrogenase flavin peptide. The excitation wavelength was 280 nm. All values are relative to tryptophan at pH 3.3.

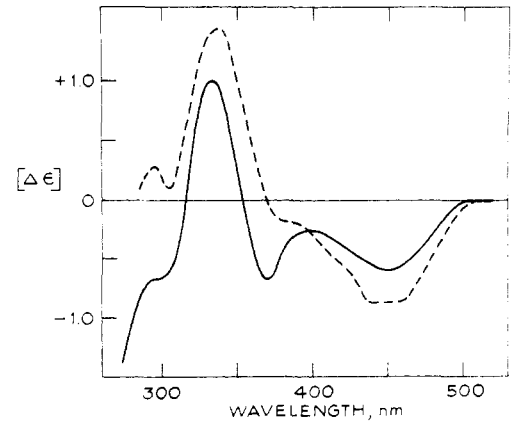

FIGURE 4: Circular dichroic spectra of the flavin peptide from β-cyclopiazonate oxidocyclase and of 8α-(N^1-histidyl)riboflavin. Flavin peptide (FMN level) (—); 8α-(N^1-histidyl)riboflavin (- - -). The compounds were dissolved in H_2O with an adjusted pH of 3.7.

comes manifest. It is noteworthy that the fluorescence of the flavin is also quenched by tryptophan in the tryptic peptide derived from succinate dehydrogenase, although the two residues are separated by some 20 amino acids (Kenney et al., 1972).

Such mutual quenching of the fluorescence of the flavin and of the aromatic amino acid present in the flavin peptide can result either from the direct molecular overlap of the aromatic ring systems, to collisional interaction, or to Förster energy transfer. The first two alternatives, although only operative over relatively short distances, cannot be a priori precluded because of spatial separation of the flavin and tryptophan residues on the *linear* sequence of the peptide, because folding might bring the aromatic rings into interacting positions. The latter, however, is operative over relatively large distances (up to 60 Å (Wehry and Roger, 1966)). Evidence for the first of these alternatives can be provided by circular dichroism only if the aromatic residues overlap in a geometrically specific rigid complex, which would then result in a large alteration of the flavin circular dichroic bands in the visible region, as shown in previous work by Miles and Urry (1968) for FAD and in this laboratory for *Chromatium* cytochrome c_{552} (Kenney et al., 1974a).

The results in Figure 4 compare the circular dichroic spectra of 8α-(N^1-histidyl)riboflavin with that of the FMN peptide from β-cyclopiazonate oxidocyclase. The spectra are quite similar in shape and intensity, although not identical. The increased dichroism shown by the peptide at 300 and 370 nm, as compared with its histidylriboflavin component, suggests some differences in the environment of the flavin. The lack of major enhancement of the optical activity, however, indicates that the flavin and tryptophan ring systems do not overlap in a geometrically specific rigid manner. Further work is necessary to ascertain whether the mutual fluorescence quenching is the result of Förster energy transfer or of collisional interaction between the aromatic moieties.

Conclusions

The demonstration that the prosthetic group of β-cyclopiazonate oxidocyclase is 8α-(N^1-His)FAD increases the list of known types of covalently bound flavin occurring in enzymes to four (Singer and Edmondson, 1974; Singer and Kenney, 1974). β-Cyclopiazonate oxidocyclase is not the only enzyme known to contain 8α-(N^1-His)FAD at its active center. We have presented evidence that the same flavin is present in a bacterial thiamine dehydrogenase (Singer and Kenney, 1974; Kenney et al., 1974b) and in L-gulono-γ-lactone oxidase from rat liver (Kenney et al., 1976).

Acknowledgments

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Characterization of Procollagen Synthesized by Matrix-Free Cells Isolated from Chick Embryo Tendons†

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ABSTRACT: The genetic type and molecular structure of the precursor forms of collagen synthesized by matrix-free tendon cells isolated from 17-day old chick embryos were examined by chromatographic and electrophoretic techniques. The [^{14}C]proline-labeled collagenous proteins secreted by the cells resolved on diethylaminoethylcellulose into two peaks, A and B. Both peaks contained type I collagenous proteins since on chromatography on carboxymethylcellulose, after limited pepsin proteolysis, both peaks contained α1 and α2 chains of collagen in a 2:1 ratio, and cyanogen bromide peptide maps of the ^{14}C-labeled protein in both peaks were similar to cyanogen bromide peptide maps derived from authentic type I collagen. Enzymatic digestion with purified mammalian collagenase demonstrated that the collagen precursor in peak B contained noncollagenous peptide extensions at both the amino- and carboxy-terminal ends of the molecule, while peak A had only carboxy-terminal extension peptides. Although both the amino- and carboxy-terminal extensions incorporated radioactive cystine, only the carboxy-terminal extensions contained interchain disulfide bonds. The carboxy-terminal extensions were also shown to incorporate radioactive tryptophan. Since most of the precursor forms of collagen recovered in the incubation medium chromatographed in peak B, it is concluded that matrix-free tendon cells secrete only type I procollagen with extension peptides at both the amino- and carboxy-terminal ends of the molecule.

The biosynthesis of collagen involves elaboration of a precursor molecule, called procollagen (for recent reviews on procollagen, see Bornstein, 1974; Miller and Matukas, 1974; Gross, 1974; Martin et al., 1975; Veis and Brownell, 1975; Uitto and Lichtenstein, 1976a; Prockop et al., 1976). The polypeptide chains of procollagen, pro-α chains, are larger than collagen α chains, because they contain additional noncollagenous peptide extensions. In studies utilizing cultured fibroblasts (Tanzer et al., 1974) and chick calvaria (Byers et al., 1975; Fessler et al., 1975), the largest form of the collagen precursors has been shown to contain extension peptides at both the amino- and carboxy-terminal ends of the molecule. In addition, collagen precursors with only amino-terminal extension peptides (Lenaers et al., 1971) and with only carboxy-terminal extension peptides (Byers et al., 1975; Fessler et al., 1975) have been isolated.

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