Inhibitors of Procollagen N-Protease. Synthetic Peptides with Sequences Similar to the Cleavage Site in the Proα1(I) Chain†

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ABSTRACT: A series of peptides was synthesized with amino acid sequences identical with the cleavage site at which the procollagen N-protease cleaves the N-terminal propeptide from the proα1 chain of type I procollagen. Peptides up to 11 residues in length did not serve as substrates for the enzyme, an observation consistent with the demonstration that the N-protease will not cleave denatured procollagen or dissociated proα chains. Several of the peptides, however, served as effective inhibitors of the cleavage of procollagen. Comparison of the inhibitor activities of peptides of varying lengths suggested that the L-phenylalanine found three residues to the left of the cleavage site was important for inhibitor activity. This suggestion was confirmed by synthesis of analogues of inhibitory peptides in which L-phenylalanine was replaced by D-phenylalanine, tyrosine, lysine, aspartic acid, or glycine.

Collagen is secreted from cells as procollagen, a larger precursor with an extension propeptide on both the N terminus and C terminus of each of the three α chains of the protein (for reviews, see Fessler & Fessler, 1978; Bornstein & Traub, 1980; Prockop et al., 1979). The primary structure of the N-propeptide on the two proα1(I) chains of type I procollagen from calf (Hörlein et al., 1978, 1979) has been determined, and a large part of the sequence of the same propeptide from sheep (Rohde et al., 1979) has been established. These proα1 N-propeptides begin with an N-terminal globular domain of 86 residues. This is followed by 45 residues with a $(Gly\text{-}X\text{-}Y)_n$ repeating structure similar to that of the α chains of collagen. Finally, there is a short globular sequence of eight residues. The C-terminal residue is proline, and the bond cleaved in the conversion of procollagen to collagen is -Pro-Gln- (Hörlein et al., 1978). Comparison of the calf and sheep N-propeptides shows a highly invariant structure. The comparable N-propeptides from other species have not been completely characterized, but those from rat (Smith et al., 1977) and chick embryos (Monson & Bornstein, 1973; Morris et al., 1975; Hoffmann et al., 1976; Olsen et al., 1976; Tuderman et al., 1978) appear similar in size to those of calf and sheep. The N-propeptide of proα1(I) from chick embryos, however, is slightly shorter than the N-propeptide from calf and sheep in that it lacks the initial glutamate-rich sequence of seven amino acids found in the calf and sheep N-propeptides (D. Pesciotta, B. R. Olsen, and P. P. Fietzek, unpublished experiments). The structure of the N-propeptide from the proα2 of type I procollagen has been partially defined. One report (Smith et al., 1977) indicates that the proα2 N-propeptide from the rat is the same size as the proα1(I) N-peptide. However, the proα2 N-propeptides from calf, sheep (Becker et al., 1976, 1977), and chick embryos (Tuderman et al., 1978) are shorter in that they lack the initial globular domain of about 100 residues found in the proα1(I) propeptide. Instead, the proα2 N-propeptide begins with a collagen-like $(Gly\text{-}X\text{-}Y)_n$ sequence similar to the collagen-like domain of the proα1 N-propeptide. Although most of the primary structure of the proα2 N-propeptide from calf is now known, the last 9 to 16 residues at the C terminus are still in doubt (D. Hörlein and P. P. Fietzek, unpublished experiments).

In the conversion of type I procollagen to collagen the three N-propeptides appear to be removed en bloc by a single procollagen N-protease (Lapière et al., 1971; Lenaers et al., 1971; Tuderman et al., 1978; Leung et al., 1979). The C-propeptides are removed by a separate enzyme (Layman & Ross, 1973; Goldberg et al., 1975; Duksin & Bornstein, 1977; Duksin et al., 1978; Leung et al., 1979). The C-protease has not been extensively characterized, but the procollagen N-protease has been purified about 300-fold from chick embryo tendons (Tuderman et al., 1978). The N-protease cleaves native procollagen but does not appear to cleave denatured procollagen or isolated proα1(I) or proα2 chains.

Materials and Methods

General Procedures for Synthesis of Peptides. Synthesis of the peptides was carried out by combining stepwise elongation and fragment condensation in solution (Figures 1 and 2). α-Amino and α-carboxyl groups were protected with Boc-[1] and B-ester groups. Functional groups in side chains of Ser, Tyr, Lys, Asp, and Glu were protected with B, BCl (Erickson & Merrifield, 1973a), Z(Cl) (Erickson & Merrifield, 1973b), and B-ester groups, respectively. All coupling reactions of fragments were carried out in DMF using the DCC–HOBt method. Finally, protecting groups of fully protected linear peptides were removed simultaneously by a single treatment with HF at 0 °C for 30 min in the presence of anisole (Sakakibara & Shimonishi, 1965; Sakakibara et al., 1967). The crude products were purified by gel filtration on Bio-Gel P2 or P4 (Bio-Rad) after treatment with Dowex 1-X4 (AcO^- form).

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[1] Abbreviations used: a, trifluoroacetic acid; B, benzyl; BCl, 2,6-dichlorobenzyl; Boc, *tert*-butyloxycarbonyl; D–b, dicyclohexylcarbodiimide and *N*-hydroxybenzotriazole; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; Dnp, 2,4-dinitrophenyl; EDTA, ethylenediaminetetraacetate; h, hydrogen and palladium/charcoal; HOBt, 1-hydroxybenzotriazole; $NaDodSO_4$, sodium dodecyl sulfate; Su, *N*-hydroxysuccinimide; Tris, tris(hydroxymethyl)aminomethane; TLC, thin-layer chromatography on silica gel; Z(Cl), 1-chlorobenzyloxycarbonyl; N-I, N-II, N-III, C-I, C-II, and C-III, intermediate peptide fragments identified in Figure 1; IV, V, VII, VIII, X, and XIII, synthetic peptides identified in Figure 2; Dnp-VII, Dnp derivative of peptide VII; Dnp-XI, Dnp derivative of the 11-residue peptide beginning with Phe and ending with Glu (see Figures 1 and 2).

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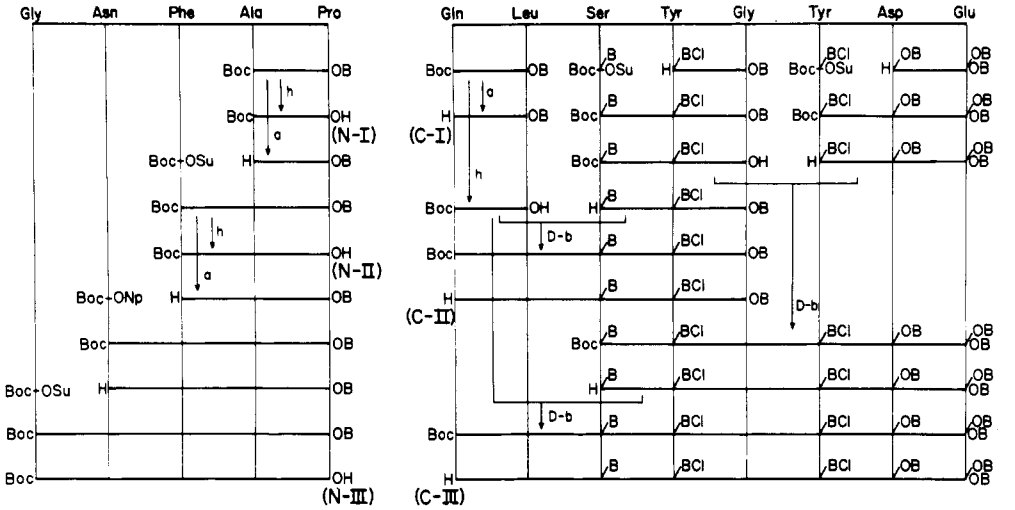

FIGURE 1: Scheme for the synthesis of intermediates. For details see Materials and Methods and Table I.

(N-I) + (C-I) → Ala-Pro-Gln-Leu IV

(N-II) + (C-I) → Phe-Ala-Pro-Gln-Leu V

(N-I) + (C-II) → Ala-Pro-Gln-Leu-Ser-Tyr-Gly VII

(N-II) + (C-II) → Phe-Ala-Pro-Gln-Leu-Ser-Tyr-Gly VIII

(N-III) + (C-II) → Gly-Asn-Phe-Ala-Pro-Gln-Leu-Ser-Tyr-Gly X

(N-II) + (C-III) + DNP → DNP-Phe-Ala-Pro-Gln-Leu-Ser-Tyr-Gly-Tyr-Asp-Glu DNP-XI

(N-III) + (C-III) → Gly-Asn-Phe-Ala-Pro-Gln-Leu-Ser-Tyr-Gly-Tyr-Asp-Glu XIII

FIGURE 2: Scheme for condensation of peptide fragments.

Dnp derivatives of peptides were prepared by the following procedures. The Boc group was removed from fully protected linear peptide by trifluoroacetic acid. The partially protected peptide was dissolved in DMF, and the pH was adjusted to 8 with triethylamine. A fivefold excess of 2,4-dinitrofluorobenzene was added, and the solution was vigorously stirred in the dark at room temperature. Protecting groups on the Dnp-peptides were then removed as above.

Substituted analogues of the peptides were synthesized by using the active ester method to add several different amino acids to the N terminus of the tetrapeptide B-ester, Ala-Pro-Gln-Leu-OB. Protecting groups were removed as described above.

Homogeneity of the final peptides, the Dnp derivatives, and the substituted analogues was confirmed by TLC, paper chromatography, paper electrophoresis, and amino acid analysis.

A total of eight peptides with sequences the same as the cleavage site in the proα1(I) chain were synthesized. Two Dnp derivatives and five peptides which were substituted analogues of peptide V were synthesized. Data on four of the synthesized peptides are presented in Table I and we present here the conditions for synthesis and characterization of three additional peptides.

Synthesis of Peptide VIII. The peptide was synthesized by condensation in solution of fragments N-II and C-II (Figures 1 and 2). N-II was homogeneous by TLC in chloroform–methanol–acetic acid (95:5:3) and ethyl acetate–benzene (1:1). Boc-C-II was homogeneous by TLC in chloroform–methanol–acetic acid (95:5:3 and 80:10:5). Boc-C-II (600 mg, 0.59 mmol) was allowed to react with 2 mL of F_3AcOH at room temperature for 30 min. F_3AcOH was removed by evaporation at reduced pressure. Ten milliliters of ether was added to the residue, and the sample was dried over NaOH in vacuo. The product was dissolved in 3 mL of DMF and neutralized with triethylamine. N-II (284 mg, 0.65 mmol) was added to the solution followed by addition of HOBt (88 mg, 0.65 mmol). The solution was cooled to −10 °C and the DCC (134 mg, 0.65 mmol) dissolved in 1 mL of DMF was slowly added. The solution was stirred at −10 °C for 3 h and then at 4 °C overnight. The reaction mixture was filtered and the filtrate was added to 50 mL of 0.5 N HCl in an ice bath. The precipitate that formed was isolated by filtration and washed with water. After drying in vacuo, the residue was reprecipitated from ethanol, ethyl acetate, and ether to yield 760 mg of product, which was dried over NaOH and concentrated H_2SO_4 in vacuo.

The fully protected octapeptide (350 mg, 0.26 mmol) was treated with approximately 5 mL of anhydrous HF at 0 °C for 30 min in the presence of 1 mL of anisole (Sakakibara & Shimonishi, 1965; Sakakibara et al., 1967). Excess HF was then removed under reduced pressure at −5 to 0 °C. The residue was dissolved in 30 mL of 10% acetic acid, and the solution was extracted with 20 mL of ether. The aqueous phase was passed through a column of Dowex 1-X4 (AcO^-). The column was eluted with 100 mL of water, and the eluant was lyophilized to yield 186 mg of product. The crude material was dissolved in a small amount of 1 M acetic acid and chromatographed on a 2.6 × 84 cm column of Bio-Gel P-2 (Bio-Rad), which was eluted with 1 M acetic acid. Fractions of 6 mL were collected. The absorbance of the fractions was assayed at 280 nm, and the major peak (fractions 39 to 44)

Table I: Characterization of Several Synthetic Peptides

	peptides			
	V	VII	X	XIII
Asp			0.89 (1)	1.78 (2)
Ser		0.88 (1)	0.82 (1)	0.87 (1)
Glu	1.06 (1)[a]	0.99 (1)	1.00 (1)	1.92 (2)
Pro	1.03 (1)	1.02 (1)	1.03 (1)	1.07 (1)
Gly		1.00 (1)	2.00 (2)	2.00 (2)
Ala	1.00 (1)	1.01 (1)	1.01 (1)	1.01 (1)
Leu	0.98 (1)	0.93 (1)	0.95 (1)	0.94 (1)
Tyr		0.99 (1)	0.99 (1)	1.92 (2)
Phe	0.98 (1)		0.97 (1)	0.98 (1)
R_f A[b]	0.44	0.15	0.17	0.17
R_f B[c]	0.65	0.42	0.50	0.36

[a] Expected value. [b] TLC in 1-butanol–acetic acid–water (4:1:1). [c] TLC in 1-butanol–acetic acid–pyridine–water (15:3:10:12).

was pooled and lyophilized to yield 134 mg of product. The product was homogeneous as tested by paper electrophoresis in 0.5 M acetic acid, in 0.2 M pyridine acetate (pH 4.8) and in 0.05 M sodium phosphate (pH 9.5) and by TLC in 1-butanol–acetic acid–water (4:1:1) and 1-butanol–acetic acid–pyridine–water (15:3:10:12). Amino acid analysis: Phe, 1.01; Ala, 1.04; Pro, 1.06; Glu, 1.01; Leu, 0.93; Ser, 0.85; Tyr, 0.97.

Synthesis of D-*Phe-Ala-Pro-Gln-Leu.* The D-Phe analogue of peptide V was synthesized as follows. The protected peptide Boc-Ala-Pro-Gln-Leu-OB (93 mg, 0.15 mmol) was reacted with 2 mL of F_3AcOH at room temperature for 20 min. F_3AcOH was removed by evaporation, and the residue was dissolved in 1 mL of DMF and neutralized with triethylamine in an ice bath. Boc-D-Phe-OSu (61 mg, 0.17 mmol) was added, and the solution was stirred at room temperature for 2 days. The reaction mixture was diluted to 50 mL with ethyl acetate and was washed with 1 N HCl, 5% $NaHCO_3$, and water. The organic phase was dried over anhydrous $MgSO_4$ and was concentrated to yield 98 mg of product, from which 88 mg was recrystallized from ethyl acetate and *n*-hexane. A single spot was obtained with TLC with chloroform–methanol–acetic acid (95:5:3). The protected pentapeptide (44 mg, 57 μmol) was treated with HF, and the product was isolated by the same procedure described for the preparation of VIII. The product was purified on a 1.6 × 86 cm column of Bio-Gel P-2 which was eluted with 0.1 M acetic acid. Fractions of 2.1 mL were collected, and the absorbance of the fractions was assayed at 230 and 260 nm. The major peak (fractions 55 to 60) was pooled and lyophilized to yield 25 mg of product. A single spot was obtained by paper electrophoresis in 0.2 M pyridine acetate (pH 4.8) and in 0.5 M acetic acid and by TLC in 1-butanol–acetic acid–water (4:1:1) and in 1-butanol–acetic acid–pyridine–water (15:3:10:12). Amino acid analysis: Phe, 0.98; Ala, 1.00; Pro, 0.99; Glu, 1.04; Leu, 0.95.

Synthesis of Dnp-XI. C-III (176 mg, 0.1 mmol) was dissolved in 2 mL of DMF, and the solution was neutralized with triethylamine. N-II (48 mg, 0.11 mmol) and HOBt (15 mg, 0.11 mmol) were added, and the solution was reacted with DCC (23 mg, 0.11 mmol) under the conditions used for preparation of VIII. After the solution was dried in vacuo, 134 mg of the fully protected undecapeptide was obtained. The product (60 mg, 29 μmol) was reacted with 1 mL of F_3AcOH at room temperature for 20 min. F_3AcOH was removed by evaporation, and 5 mL of ether was added to yield a precipitate which was dried over NaOH in vacuo. This product was dissolved in 1 mL of DMF and the pH was adjusted to 8 with triethylamine. 2,4-Dinitrofluorobenzene (28 mg, 0.15 mmol) was added, and the solution was vigorously stirred in the dark at room temperature for 1 day. The reaction mixture was added to 10 mL of ether, and the precipitate was separated by centrifugation at 3000 rpm for 5 min. The precipitate was washed with ether and then dried in vacuo. The product was treated with HF and isolated by the same procedure described for the preparation of VIII. The peptide was then purified on a 1.5 × 100 cm column of Bio-Gel P-4 which was eluted with 50% acetic acid. The fractions of 3.5 mL were collected, and the absorbance of the fractions was measured at 280 and 365 nm. The major peak (fractions 34 to 37) was pooled and lyophilized. The intermediates and the peptide were protected from light throughout the synthesis. A single spot was obtained by TLC in 1-butanol–acetic acid–water (4:1:1). Amino acid analysis: Ala, 1.16; Pro, 1.02; Glu, 2.12; Leu, 0.94; Ser, 0.80; Tyr, 1.97; Gly, 1.25; Asp, 1.57.

Assay of Procollagen N-Protease. Procollagen N-protease was partially purified from leg tendons of 17-day-old chick embryos with the procedures described previously (Tuderman et al., 1978). Because the enzyme obtained after the final gel filtration step was unstable, we used here the 60-fold purified enzyme obtained from the previous step of affinity chromatography on concanavalin A–Sepharose. The enzymic reaction was carried out in 100 μL containing 0.4 unit (5 to 10 μL) of enzyme solution, 0.15 M NaCl, 5 mM $CaCl_2$, and 0.1 M Tris-HCl buffer (pH 7.4 at 30 °C). The reaction was carried out for 24 h at 30 °C. To assay the peptides as substrates, they were added to the reaction mixture in amounts varying from 0.1 to 0.5 mg, and aliquots were assayed by TLC. To assay the peptides as inhibitors, 5 μg of ^{14}C-labeled chick embryo procollagen (about 10 000 cpm/μg) was used as a substrate for the reaction, and the reaction was terminated with 50 μL of 0.5 M EDTA, 100 μL of fetal calf serum, and 750 μL of 40% saturated ammonium sulfate (243 mg/mL). After 1 h at room temperature, the sample was centrifuged at 15000*g* for 30 min, and a 0.5-mL aliquot of the supernate was counted to assay the ^{14}C-labeled N-propeptide released by the enzyme. In occasional experiments the specificity of the reaction was verified (Tuderman et al., 1978) by examining aliquots of the total reaction mixture by polyacrylamide gel electrophoresis in $NaDodSO_4$.

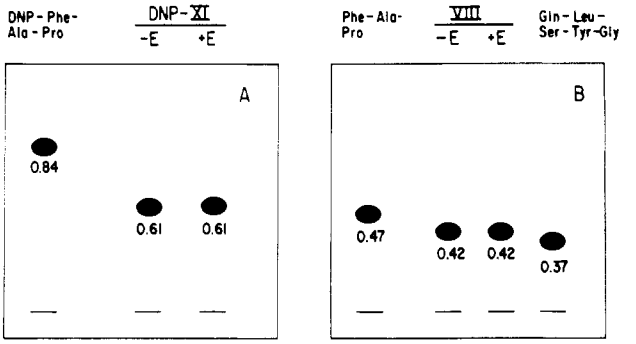

FIGURE 3: Failure of procollagen N-protease to cleave Dnp-XI and VIII. The peptides were incubated with the enzyme as described in the text. TLC was carried out in 1-butanol–acetic acid–water (4:1:1): (A) reaction products with Dnp-XI after incubation with and without enzyme; (B) reaction products with VIII after incubation with and without enzyme.

Amino Acid Analysis. Samples were hydrolyzed in 6 N HCl in the presence of phenol. The tubes were sealed in vacuo and heated at 116 °C for 18 h. The analyses were kindly carried out for us by Alex G. Georgiades at the Roche Institute of Molecular Biology on a Durrum D-500 analyzer.

Results

Assay of Peptides as Substrates. The first question examined here was whether peptides with the same sequence as the cleavage site in the proα1(I) chain were substrates for procollagen N-protease.

Dnp-XI in a concentration of 1 mg/mL (0.69 mM) was reacted with 0.4 unit of procollagen N-protease for 24 h, and the reaction was assayed by TLC. As shown in Figure 3A, there was no indication that Dnp-XI was cleaved to the expected product Dnp-Phe-Ala-Pro. Similar results were obtained when Dnp-VII was tested in a concentration of 1 mg/mL (1.1 mM) under the same conditions. Standardizations of the TLC system with varying amounts of Dnp-Phe-Ala-Pro suggested that a 5% cleavage of the peptides to the expected fragments would have been detected.

Since the Dnp group on Dnp-XI may have prevented cleavage of the peptide, similar experiments were carried out with VIII. The peptide was incubated at a concentration of 1 mg/mL (1.13 mM) under the same conditions used with

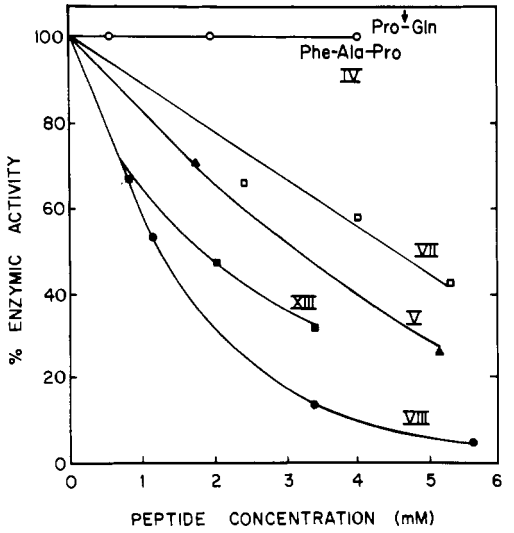

FIGURE 4: Inhibition of procollagen N-protease by synthetic peptides. As described in the text, ^{14}C-labeled procollagen was used as the substrate.

Dnp-XI. As shown in Figure 3B, there was no cleavage of VIII to the expected products Phe-Ala-Pro and Gln-Leu-Ser-Tyr-Gly under the conditions in which cleavage of 5% of the peptides would have been detected.

Assay of Peptides as Inhibitors. Since the peptides Dnp-VII, Dnp-XI, and VIII were not substrates, these and related peptides (Figure 2) were assayed as inhibitors of the N-protease reaction. The peptides were added to the enzymic reaction in concentrations varying from 1 to 5 mg/mL (0.6 to 5.8 mM) and inhibition of the cleavage of ^{14}C-labeled chick embryo procollagen was assayed. As indicated in Figure 4, the short peptides Pro-Gln, Phe-Ala-Pro, and Ala-Pro-Gln-Leu did not have any inhibitor activity. In contrast, the peptides V, VII, VIII, and XIII all inhibited the reaction. Peptides VIII and XIII were somewhat more effective than the other two, and 50% inhibition was obtained with 1 to 2 mM concentrations of VIII and XIII. Peptide VIII appeared to be slightly more effective than XIII, but the difference might be explained by the observation that XIII was somewhat less soluble in neutral buffer. The peptide X proved to be too insoluble in neutral buffer to assay.

Substituted Analogues of Peptide V as Inhibitors. As indicated in Figure 4, peptide V was a relatively good inhibitor, whereas peptide IV did not show any inhibitor activity. Also, peptide VIII was a better inhibitor than peptide VII. Since peptides V and VIII contained L-phenylalanine three residues to the left of the cleavage site whereas the other two peptides did not, these observations suggested that the L-phenylalanine residue was important in determining inhibitor activity of the peptides. To test this possibility further, a series of analogues was synthesized in which L-phenylalanine in V was replaced by one of several amino acids. As indicated in Table II, replacement of L-phenylalanine with D-phenylalanine markedly reduced the inhibitor activity. Similar results were obtained when the L-phenylalanine was replaced with tyrosine, glycine, lysine, or aspartic acid.

Table II: Effect of Replacing L-Phenylalanine on Inhibitor Activity of Synthetic Peptides[a]

peptide (mg/mL)	inhibiton (%)					
	V	D-Phe[b]	Tyr	Gly	Lys	Asp
1	42	17	12			8
3	86	17	18	0	16	6
5	100			0	22	

[a] Reaction was assayed with ^{14}C-labeled procollagen as substrate. [b] Amino acid inserted into V in place of L-phenylalanine.

Discussion

The strategies employed here for peptide synthesis were selected to minimize the risk of racemization. In particular, the majority of the fragment condensations was carried out with fragments in which proline was at the C terminus. The remaining condensation steps were carried out either with active esters or with a carbodiimide procedure in which racemization is minimal (König & Geiger, 1970). The peptides obtained were homogeneous by electrophoresis and TLC, and most were soluble under neutral conditions appropriate for assaying the procollagen N-protease.

The peptides Dnp-VII, Dnp-XI, and VIII were shown not to be substrates for the enzyme even under conditions in which a 5% cleavage could have been detected. The failure of these peptides to serve as substrates is not surprising, since the enzyme will not act on procollagen which is denatured or separated into proα1 and proα2 chains (Tuderman et al., 1978). Most of the peptides larger than the tetrapeptide IV, however, served as inhibitors of the enzyme. Inhibition of the enzyme by these peptides is consistent with the observation that isolated calf pNα1 chains at a concentration of 1 μM inhibited the reaction by about 50% (unpublished observation). The higher concentrations of synthetic peptides required for the same degree of inhibition may well be explained by their shorter length. Comparing the peptides synthesized here demonstrated that the presence of L-phenylalanine in the third position to the left of the cleavage site was critical, since systematic substitution of the phenylalanine with several amino acids markedly decreased the inhibition activity.

The peptides have proved useful in further purification of procollagen N-protease by affinity chromatography (L. Tuderman, T. Morikawa, O. Helle, and D. J. Prockop, unpublished experiments). It seems possible that peptides which are substrates could be prepared if the peptides were elongated to include the collagen-like sequences such as those found toward the N terminus of the cleavage site in proα1(I). This might be particularly true if a long enough sequence of $(Pro\text{-}Hyp\text{-}Gly)_n$ were included to allow the peptides to associate into a triple-helical conformation.

Acknowledgments

The authors gratefully acknowledge the expert assistance of Karen Gilbert.

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Effect of Hydrophobic Carboxyl Reagents on the Proton Flux through Coupling Factor CF_0 in Thylakoid Membrane†

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ABSTRACT: Specific carboxyl reagents, 1-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ) and 1-(isobutoxycarbonyl)-2-isobutoxy-1,2-dihydroquinoline (IIDQ), were found to interact with coupling factor CF_0 of spinach chloroplasts in a way similar to dicyclohexylcarbodiimide (DCCD). At a concentration of 50 μM, both compounds reduced proton leakage out of chloroplasts which were deficient in coupling factor CF_1. The rate of resealing the uncoupled chloroplasts by EEDQ and IIDQ was ~20 times slower than that by DCCD. The regained proton accumulation under constant illumination was 95, 75, and 45% of control chloroplasts for DCCD, IIDQ, and EEDQ, respectively. This is in the same order as the sizes of the resulting groups attached to CF_0. Reaction with EEDQ prior to the addition of [^{14}C]DCCD reduced the incorporation of radioactivity into the DCCD-binding protein in chloroplasts. These compounds at low concentration (50 μM) inhibited photophosphorylation and heat-activated ATPase activity of control chloroplasts but had little effect on either the electron flow from water to 2,6-dichlorophenolindophenol or the ATPase activity of CF_1 extracted into solution. Light-induced proton uptake of control chloroplasts at pH 8.0 was stimulated to different degrees by these reagents also in the order DCCD > IIDQ > EEDQ; however, this effect was not observed at pH 6.6. These results suggest that these hydrophobic carboxyl reagents interact specifically and competitively with the same site in CF_0, presumably a reactive carboxyl group.

The free energy change due to light-driven electron transport in chloroplasts is coupled to ATP formation by the coupling factor complex CF_1·CF_0.[1] The complex has been resolved into the soluble ATPase (CF_1), which provides the catalytic site for an adenine nucleotide, and a membrane-integrated part (CF_0) to which CF_1 is attached. The isolated CF_1 is composed of five subunits, which are denoted by α, β, γ, δ, and ϵ, respectively, in the order of decreasing molecular weight and have a suggested stoichiometry of 2:2:1:1:2 (Baird & Hammes, 1976). CF_0 probably consists of three or four subunits (Pick & Racker, 1979), and it is involved in the transport of protons across the thylakoid membrane.

Investigations of CF_1·CF_0 have been reviewed by Baird & Hammes (1979), McCarty (1977), and Nelson (1976). The CF_1·CF_0 complex acts as a proton channel which utilizes the proton gradient generated by electron transport to synthesize ATP (Mitchell, 1961, 1966). It is well-known that CF_1-depleted chloroplasts obtained by EDTA washing or NaBr treatment lack the ability to accumulate protons under illumination. However, the proton uptake can be restored by reconstitution of purified CF_1 (Lynn & Straub, 1969) or by

† From the Bioenergetics Laboratory, State University of New York, Buffalo, New York 14214. *Received December 19, 1979.* This work was supported in part by grants from the National Science Foundation (PCM 7715002) and by the National Institute of General Medical Sciences, U.S. Public Health Service (GM-19990).

[1] Abbreviations used: Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; EEDQ, 1-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; IIDQ, 1-(isobutoxycarbonyl)-2-isobutoxy-1,2-dihydroquinoline; EDTA, ethylenediaminetetraacetic acid; DCIP, 2,6-dichlorophenolindophenol; DTT, dithiothreitol; CF_1·CF_0, the complex of chloroplast coupling factors; Dio-9, an antibiotic of unknown structure (McCarty et al., 1965); $NaDodSO_4$, sodium dodecyl sulfate; PEI, poly(ethylenimine).

0006-2960/80/0419-2650$01.00/0