Hill, T. L. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4918-4922.
Jordan, J., Ackerman, E., & Berger, R. L. (1956) *J. Am. Chem. Soc. 78*, 2979-2983.
Lamm, O., & Sjostedt, G. (1938) *Trans. Faraday Soc. 34*, 1158-1163.
McKinnie, R. E., & Olson, J. S. (1981) *J. Biol. Chem. 256*, 8928-8932.
Noyes, R. M. (1961) *Prog. React. Kinet. 1*, 129-160.
Roughton, F. J. W. (1959) *Prog. Biophys. Biophys. Chem. 9*, 55-104.
Samson, R., & Deutch, J. M. (1978) *J. Chem. Phys. 68*, 285-290.
Schurr, J. M., & Schmitz, K. S. (1976) *J. Phys. Chem. 80*, 1934-1936.
Shoup, D., Lipari, G., & Szabo, A. (1981) *Biophys. J. 36*, 697-714.
Strother, G. K., Ackerman, E., Anthony, A., & Strickland, E. H. (1959) *Am. J. Physiol. 196*, 517-519.
von Smoluchowski, M. (1917) *Z. Phys. Chem., Stoechiom. Verwandtschaftsl. 92*, 129-168.
Weller, A. (1961) *Prog. React. Kinet. 1*, 189-233.

Kinetics of Reduction of the Intersubunit Disulfides of the Carboxyl Propeptide of Type I Procollagen†

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ABSTRACT: The carboxyl propeptide produced by proteolysis during maturation of type I procollagen to collagen was purified to homogeneity from the medium of cultured chick embryo calvaria by a new method. The propeptide was identified as such by its amino acid composition and migration pattern through sodium dodecyl sulfate-polyacrylamide gels in the absence and presence of dithiothreitol. Reduction of the intersubunit disulfides, which covalently join the two C1 and one C2 polypeptides of the carboxyl propeptide, was studied by incubating the propeptide in the presence of dithiothreitol for various times under nondenaturing conditions at pH 8.2. The reduction process was characterized by the appearance of disulfide-linked dimers. The appearance of dimers correlated with the disappearance of the carboxyl propeptide. Monomers, retaining intrasubunit disulfides, appeared concomitant with dimer formation. Reduction of the intersubunit disulfides of the dimers followed; intrasubunit disulfides were retained. The rate of the first process, trimer to dimer plus monomer, was an order of magnitude larger than the rate for the second process, dimer to monomers. The dimeric intermediates were composed of approximately equivalent amounts of $(C1)_2$ and (C1, C2). The kinetics of formation and reduction of $(C1)_2$ and (C1, C2) could not be differentiated by the techniques used. The relative amounts of intermediates found were not those expected if quasi-equivalent intersubunit disulfides were reduced in a random fashion. A possible model for reduction of the intersubunit disulfides of the propeptide has been proposed, and implications for the intersubunit polypeptide surface contacts have been discussed.

The carboxy-terminal extension of the several pro α[1] chains of the procollagens has been strongly implicated as the site for pro α chain association during collagen maturation (Schofield et al., 1974; Harwood et al., 1976, 1977; Rosenbloom et al., 1976; Kao et al., 1979; Bächinger et al., 1980; Gerard et al., 1981). Little is known concerning the conformation and other possible functions of the carboxy-terminal extension; however, the amino acid sequences of the type I extensions have been deduced from nucleotide sequences of cDNA clones (Fuller & Boedtker, 1981). Unlike the amino-terminal extension which can be obtained in large yield from skins of dermatosporatic animals (Becker et al., 1976; Engel et al., 1977), carboxy-terminal extensions are not readily available; only the carboxyl propeptide derived from type I procollagen has been obtained in reasonable quantities (Olsen et al., 1977). This carboxyl propeptide, formed by proteolysis during the maturation of type I procollagen to collagen, appears to be potentially useful for studying processes involved in the folding of polypeptides and in polypeptide chain association for reasons outlined below.

Both type I collagen and its biosynthetic precursor, type I procollagen, are composed of two genetically distinct, but related, chains which associate in a 2:1 ratio as $[\alpha1(I)]_2\alpha2$ and [pro $\alpha1(I)]_2$ pro $\alpha2$, respectively. This ratio predominates in vivo, but a procollagen and a collagen composed of only a single polypeptide ([pro $\alpha1(I)]_3$) and $[\alpha1(I)]_3$ have also been identified (Mayne et al., 1976; Jimenez et al., 1977; Little et al., 1977; Wohllebe & Carmichael, 1978; Crouch & Bornstein, 1978; Smith & Niles, 1980). On the other hand, neither [pro $\alpha2]_3$ nor $[\alpha2]_3$ has ever been identified in vivo. Further, in tissues or cell cultures producing more than one type of collagen, heterogeneous mixtures of genetically distinct collagen

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[1] Abbreviations: pro α, intact polypeptide chain of procollagen; pC-collagen, procollagen lacking the amino-terminal extensions; carboxyl propeptide, disulfide-linked carboxy-terminal extensions derived from proteolysis during maturation of type I procollagen to collagen; C1 and C2, individual polypeptide chains of the carboxyl propeptide without reference to the redox state of the chains' cysteines; T and T′, trimeric states of the carboxyl propeptide visualized on polyacrylamide gels (T′ is an open form of T); D and M, dimeric and monomeric states, respectively, of the carboxyl propeptide visualized on polyacrylamide gels (both species retain intrasubunit disulfides, and D retains intersubunit disulfides also); Con A, concanavalin A; DEAE, diethylaminoethyl; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; TPE buffer, 0.1 M Tris-PO_4, pH 8.2, 1.0 M K_2HPO_4, and 5 mM EDTA; Tris, tris(hydroxymethyl)aminomethane.

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chains are not found. Thus, there appear to be severe restrictions on collagen chain association in vivo which should be due to complementary recognition of specific structural features.

The polypeptides, C1 and C2, of the carboxyl propeptide of type I procollagen are of sufficient size, each with an approximate molecular weight of 27 500 exclusive of carbohydrate, to maintain folded structural domains independent of the remainder of the pro α chain. C1 and C2 have high sequence homology (about 60%) (Fuller & Boedtker, 1981). It is also understood that both intra- and intersubunit disulfide cross-links exist. The identity of the disulfides has not been totally established, but Olsen et al. (1977) have shown that there are approximately equal numbers of intra- and intersubunit disulfides. The sequence positions of the cysteines of C1 and C2 are strictly homologous with the exception that a single cysteine is absent in C2, suggesting that disulfides within and between the polypeptides may also be homologous. C1 has eight cysteines and C2, seven (Fuller & Boedtker, 1981).

As the carboxy-terminal extensions are believed to direct chain association in vivo, insights into this mechanism may be gained by examining association or dissociation phenomena of the isolated carboxyl propeptide in vitro under controlled redox conditions. Although the exact disulfide pairs have not been established, the relative redox potentials of the various disulfides may be useful, as they could be artificially controlled during the course of refolding–reassociation or dissociation–unfolding reactions. In conjunction with control of the redox state, alkylation of thiol groups, preventing additional disulfide–thiol exchange, allows the kinetics and intermediates of refolding–reassociation or dissociation–unfolding to be monitored (Saxena & Wetlaufer, 1970; Creighton, 1978). We have therefore undertaken a study of the carboxyl propeptide of type I procollagen mindful of the above. We report a purification procedure for the carboxyl propeptide and a study of the reduction of the disulfides of the propeptide under conditions where apparently only intersubunit disulfides are reduced. The kinetics of this process are reported and the intermediate species identified.

Experimental Procedures

Organ Culture Conditions. Upon dissection, calvaria from 17-day-old chick embryos were placed in a culture medium consisting of Dulbecco's modified Eagle's medium (MEM) [Grand Island Biological Co. (GIBCO)] buffered with 3.7 g/L $NaHCO_3$ and containing 100 units/mL penicillin G (GIBCO), 100 μg/mL streptomycin sulfate (GIBCO), 1% nonessential amino acids (MEM; GIBCO), 1% sodium pyruvate (MEM; GIBCO), 150 μg/mL sodium ascorbate (Sigma Chemical Co.), and 150 μg/mL β-aminopropionitrile fumarate (+99% purity; Aldrich Chemical Co.). Calvaria were then washed aseptically with medium of identical composition and placed in sterile 75-cm^2 plastic flasks standing upright. Sterile medium was added to each flask at a concentration of 0.5 mL/calvarium with no more than 160 calvaria per flask. A mixture of ^{3}H-labeled amino acids (New England Nuclear Co.) was added at a concentration of 1 μCi/calvarium. Each flask was thoroughly flushed with 5% CO_2–95% air, tightly capped, and placed on a slowly rotating platform at 37 °C for 24 h.

After the incubation period, spent medium was decanted and replaced with fresh medium, omitting only the ^{3}H-labeled amino acids. Organ culture was repeated for a second 24-h period. At the end of this period, spent medium was replaced for a third and final time.

Medium Treatment. After each incubation period, spent medium was treated as follows: protease inhibitors were added at final concentrations of 10^{-3} M *p*-(chloromercuri)benzoate (PCMB; Sigma), 10^{-5} M phenylmethanesulfonyl fluoride (PMSF; Sigma), 0.2–0.3 μg/mL pepstatin A (Sigma), and 20 mM EDTA. The medium was centrifuged at 12000*g* for 1 h at 4 °C and the pellet discarded. All further manipulations were carried out at 4 °C. Clarified medium was concentrated overnight by dialysis vs. poly(ethylene glycol) 20 000 (30% w/v) and then dialyzed vs. 50 mM Tris-HCl, pH 8.0 (25 °C), 10^{-4} M PCMB, and 10^{-7} M PMSF.

Column Chromatography. After thorough dialysis, the media from the three incubation periods were pooled and adjusted to a final concentration of 50 mM Tris-HCl, pH 8.0, and 2 M urea by addition of 2.0 M Tris-HCl, pH 8.0, 10 M urea (ultrapure; Schwarz/Mann), and water. This solution was applied to a 2.3 × 10 cm DEAE-cellulose column (DE-52; Whatman Ltd.) equilibrated with 50 mM Tris-HCl, pH 8.0, and 2 M urea at 4 °C. The carboxyl propeptide was eluted with a linear 0.0–0.2 M NaCl gradient, total volume of 600 mL, by a modification of the procedure of Smith et al. (1972).

The fractions containing the carboxyl propeptide were pooled and added to a buffer containing 50 mM Tris-HCl, pH 8.0, 2 M urea, 2.5 M NaCl, 5 mM $CaCl_2$, 5 mM $MnCl_2$, and 5 mM $MgCl_2$ in the final ratio of 4:1. This solution was applied to a concanavalin A–Sepharose (Con A–Sepharose; Pharmacia Inc.) column (1.1 × 23 cm) by rapid gravity flow, equilibrated with 50 mM Tris-HCl, pH 8.0, 2 M urea, 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 1 mM $MgCl_2$. Five-milliliter fractions were collected. After the sample was applied, the column was washed with slightly more than one column volume of the starting buffer and then with the same buffer prepared without urea. The carboxyl propeptide was eluted with 50 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 1 mM $MgCl_2$, and 0.5 M methyl α-D-mannoside (type III; Sigma) at a flow rate of 1–2 mL/h.

The propeptide fraction was desalted on a 4.5 × 28 cm Bio-Gel P-2 (Bio-Rad Laboratories) column equilibrated with 0.2 M ammonium bicarbonate and then lyophilized. At this stage, the carboxyl propeptide was judged 98% pure by Na-DodSO$_4$–polyacrylamide gel electrophoresis (see below). Contaminants appeared to be procollagen or pC-collagen on the basis of their electrophoretic mobility. Final purification was achieved by chromatography on a 1.5 × 90 cm Bio-Gel A-1.5 (Bio-Rad) column equilibrated with 0.2 M ammonium bicarbonate. The purified propeptide was stored lyophilized at 0 °C.

Amino Acid Analysis. Samples of the carboxyl propeptide (approximately 50 000 cpm/sample) were dissolved in 1 mL of 0.1 M Tris-HCl, pH 8.2, 8 M urea, and 1 mM EDTA, to which 0.1 mL of 0.05 M dithiothreitol (DTT; Sigma) in the same buffer was added. Samples were incubated at room temperature for 2 h followed by addition of 0.1 mL of 0.5 M iodoacetic acid (Sigma) neutralized with NaOH. Alkylation proceeded for 1 h at room temperature in the dark. The samples were desalted over Bio-Gel P-2 and lyophilized in hydrolysis tubes. Three milliliters of constant-boiling HCl (Pierce Chemical Co.) and 2 μL of 2-mercaptoethanol were added to each sample, which was then thoroughly degassed under reduced pressure. Hydrolysis proceeded for 20 h at 110 °C. A Beckman 119 CL automated amino acid analyzer was used for the analyses.

Concentration Determinations. Aliquots of all carboxyl propeptide solutions were assayed by using a Packard 3255 scintillation counter. The specific activity (dpm/milligram)

was determined from amino acid analysis by using a mean residue weight of 112. The molarity of the solutions was determined by using the specific activity and a molecular weight of 82 572 exclusive of carbohydrate content (Fuller & Boedtker, 1981). The efficiency of the scintillation counter was determined by employing a known ^3H standard. Quenching of the solutions was examined by addition of 10 μL of ^3H_2O to each sample and to a blank and by recounting. Quenching was negligible.

NaDodSO$_4$–Polyacrylamide Gel Electrophoresis. NaDodSO$_4$–10% polyacrylamide slab gels [method of Laemmli (1970)] run at 40 mA/gel were used to monitor the purification of the carboxyl propeptide. Samples of the propeptide were electrophoresed in the absence and presence of 7.5 mg/mL DTT contained in the sample buffer. Gels were stained with 2.3% Coomassie Brilliant Blue R (Bio-Rad), 46% methanol, and 10% acetic acid and destained in 10% methanol and 7% acetic acid. For measurement of the intensities of the stained protein bands, the gel lanes were excised, and a Gilford-modified Beckman DU spectrophotometer equipped with a gel scanning device was used. The areas of the densitometer tracings were integrated with a polar planimeter. Dilution studies indicated that dye binding was proportional over the concentration range measured (0–10 μg).

Kinetic Study of the Selective Reduction of the Carboxyl Propeptide. Selective reduction of the intersubunit disulfides of the propeptide was initiated by addition of 45 μL of DTT (at one of two concentrations) in a degassed solution of 0.1 M Tris–PO$_4$, pH 8.2, 1.0 M K_2HPO_4, and 5 mM EDTA (TPE buffer) to 0.45 mL of the carboxyl propeptide (about 3.6 μM) in TPE buffer at room temperature. DTT had been dried at room temperature under vacuum and, when assayed by the method of Ellman (1959), found to contain 100% of the expected thiol groups. After initiation of the reduction, 20-μL aliquots were removed at various times and added to 20 μL of 0.2 M iodoacetate in 50 mM Tris–OH, pH 6.8, terminating the reduction. After incubation in the dark for 1 h, 60 μL of a NaDodSO$_4$ sample buffer containing 0.084 M Tris-HCl, pH 6.8, 1.67% NaDodSO$_4$, 1.67% glycerol, and 0.005% bromophenol blue was added to each aliquot. The samples were stored at 4 °C and heated at 37 °C for 1 h just prior to electrophoresis. Fifty microliters of each sample was electrophoresed on NaDodSO$_4$–10% polyacrylamide gels. The amounts of trimeric (T plus T′), dimeric (D), and monomeric (M) forms of the protein at each time point were determined by densitometry and normalized to 100% of the total Coomassie Blue stain.

C1 and C2 [and separately, the two disulfide cross-linked species $(C1)_2$ and (C1, C2)] comigrated upon electrophoresis after partial reduction under the conditions outlined above, as will be shown later. Therefore, for determination of the relative quantities of each monomeric or dimeric species, individual protein bands, containing either D or M, were cut out of the gel, dialyzed against running buffer for 1 h, and reelectrophoresed, in the presence of NaDodSO$_4$ sample buffer containing 15 mg/mL DTT, into a 10% polyacrylamide gel with a 5% stacking gel at 5 mA/gel. The ratio of C1 to C2 for each dimeric or monomeric species was determined by densitometry.

Results and Discussion

Purification of the Carboxyl Propeptide. The carboxyl propeptide derived from type I procollagen was purified from the medium of cultured chick embryo calvaria by combination of DEAE-cellulose, Con A–Sepharose, and Bio-Gel A-1.5 chromatography. Identification of the propeptide, as such, depended on (1) the amino acid composition of the purified polypeptide (Table I), which was nearly identical with that determined indirectly from nucleotide sequences of cDNA clones coding for the carboxy-terminal regions of the pro α1(I) and pro α2 genes (Fuller & Boedtker, 1981), or amino acid analyses determined after other methods of purification (Murphy et al., 1975; Olsen et al., 1977), and (2) the migration patterns of the protein, in the absence and presence of DDT, through a polyacrylamide gel (Figure 1). Further, in the presence of DTT, the ratio of C1 to C2 was 1.90 ± 0.08, as expected.

Table I: Amino Acid Composition of the Carboxyl Propeptide

amino acid	residues/mol of protein	
	b	*c*
Asp[a]	96	96
Thr	61	64
Ser	38	38
Glu[d]	77	77
Pro	35	32
Gly	64	58
Ala	50	46
Val	32	32
$^1/_2$-Cys[e]	19	23
Met	13	17
Ile	37	42
Leu	54	51
Tyr	25	26
Phe	23	23
Lys	48	51
His	16	16
Arg	31	30
Trp[f]	19	15

[a] Sum of Asp and Asn. [b] This work. Based on a molecular weight of 82 572. The values were rounded to the nearest whole number. [c] Work of Fuller & Boedkter (1981). [d] Sum of Glu and Gln. [e] Determined as (carboxymethyl)cysteine. [f] Determined by the method of Bencze & Schmid (1957).

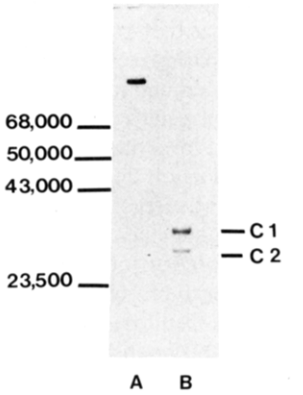

FIGURE 1: Electrophoretic profile of the carboxyl propeptide, (A) unreduced and (B) fully reduced. The positions of C1 and C2 are indicated in (B). Positions of molecular weight standards are shown: 68 000, bovine serum albumin; 50 000, immunoglobulin G, heavy chain; 43 000, ovalbumin; 23 500, immunoglobulin G, light chain.

Approximately 15–20 mg of the propeptide was obtained from the calvaria of 50 dozen embryos. Although this yield was somewhat less than that reported by Olsen et al. (1977), here, the use of high concentrations of urea was avoided. Preliminary data, not presented, suggested that our purification procedure could also be used for culture medium of chick embryo tendons.

The polypeptides of the carboxyl propeptide each have one high mannose type carbohydrate chain (Olsen et al., 1977; Clark, 1979; Pesciotta et al., 1981). To our knowledge, Con A–Sepharose affinity chromatography, in the absence of de-

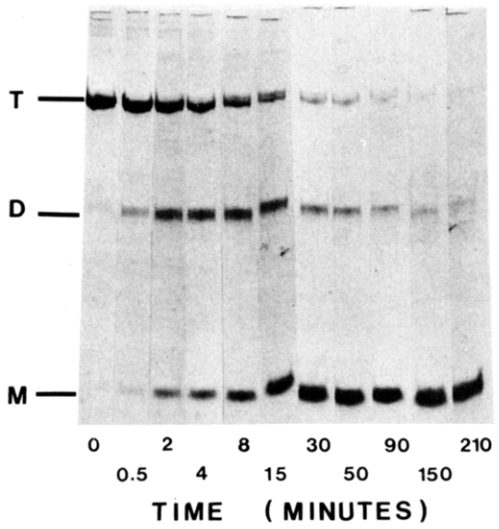

FIGURE 2: Electrophoretic separation of the species trapped by alkylation during the reduction of the intersubunit disulfides of the carboxyl propeptide by 0.5 mM DTT. The positions of T, D, and M are indicated. Note that T′ migrates slightly above T. Times at which reduction was terminated are indicated.

naturants, has not been successfully used in the isolation of either the carboxyl propeptide or procollagen. The displacement by mannose and the complete recovery of the propeptide from Con A–Sepharose were possible with 24–36 h of slow continuous elution.

Reduction of the Carboxyl Propeptide Using Nondenaturing Conditions. Sequential reduction of the carboxyl propeptide disulfides could be monitored by $NaDodSO_4$ gel electrophoresis if all thiol groups had first been scavenged by alkylation with excess iodoacetate. Figure 2 illustrates a representative sample of the different species that existed during the reduction period. As shown in that figure, the carboxyl propeptide (T) was reduced by 0.5 mM DTT in TPE buffer and was replaced over a period of 3–3.5 h by other species. On the basis of the migration of molecular weight standards (not shown), a second species (D) migrated at a position consistent with that of a dimer. The appearance and disappearance of D were also consistent with the behavior expected if reduction of the propeptide to individual polypeptides proceeded through a dimeric intermediate. A third species (M) migrated more rapidly than either C1 or C2 under conditions where both the intra- and intersubunit disulfides of the carboxyl propeptide had been reduced (e.g., Figure 1, lane B). With time, M accumulated, until it was the principal species present. As shown below, D is composed of two types of dimers with the compositions $(C1)_2$ and (C1, C2), and M is a mixture of C1 and C2 which have retained intrasubunit disulfides. As denaturing conditions were used in the electrophoretic sample buffer, we could not distinguish whether the dissociation of the polypeptides was a result of the reduction of intersubunit disulfides, alkylation, or exposure to $NaDodSO_4$.

During the course of reduction, an additional component (T′) was detected, migrating slightly above the position of the carboxyl propeptide (T). (See time points 8, 15, and 30 min in Figure 2 for clear examples of T′. In fact, T′ was the only species detected at the late times.) T′ was never observed when the propeptide was electrophoresed in the absence of DTT. Densitometer tracings indicated that the amount of T′ was nearly constant over much of the reaction period, suggesting as a first step the conversion of the carboxyl propeptide to a less compact trimeric conformation. T′ might then result as a consequence of intersubunit disulfide reduction and be an open trimeric species where two of the polypeptides are not disulfide cross-linked to each other. Alternatively, T′ may be a species where at least one intrasubunit disulfide had been reduced, although for reasons discussed later, this possibility seems less likely.

Small amounts of higher molecular weight species (less than 10% of the total protein) were often found prior to the start of, and disappeared during the course of, the reduction. These bands were likely due to some aggregation and cross-linking which occurred during storage of the propeptide. Their presence was ignored.

Kinetics of Reduction. Figure 3 expresses the percent distribution of T plus T′, D, and M as a function of time when reduction was carried out in the presence of 0.5 mM DTT at room temperature. Semilog plots of the data shown in Figure

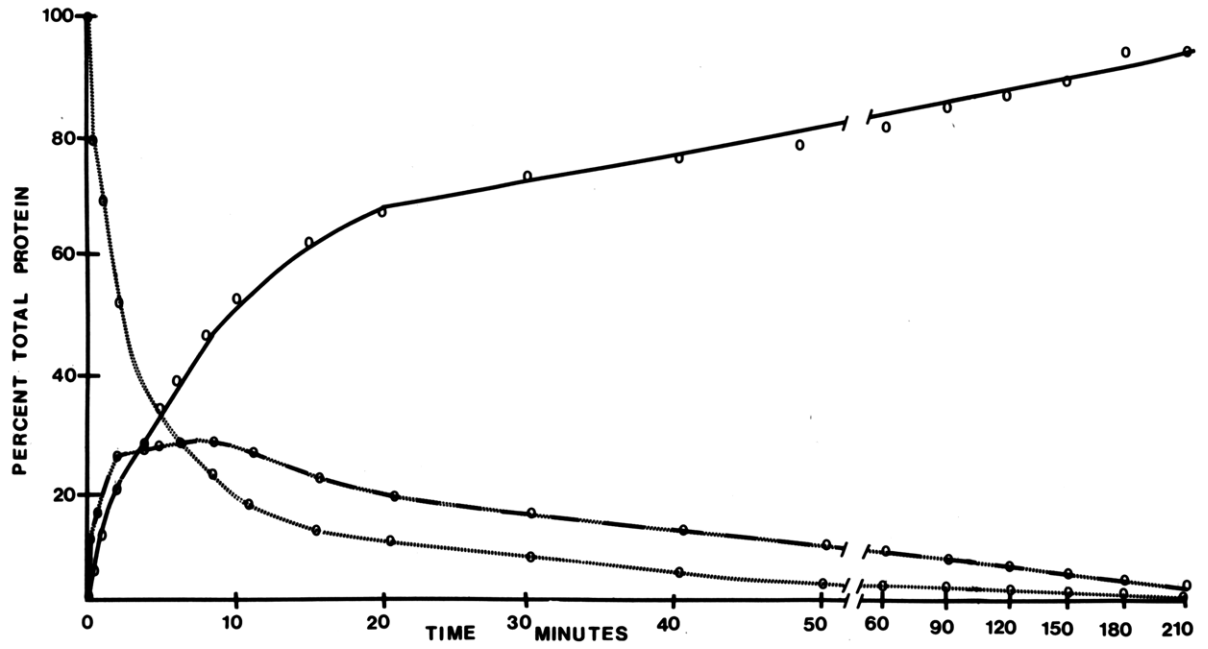

FIGURE 3: Disappearance of T plus T′ (O···O), appearance and disappearance of D (O–···O), and appearance of M (O—O) expressed as the percentage of total protein vs. time of alkylation. The values were determined from densitometry tracings of $NaDodSO_4$–polyacrylamide gels similar to those shown in Figure 2. Lines are included as an aid to visualization only. The results are the average of three experiments.

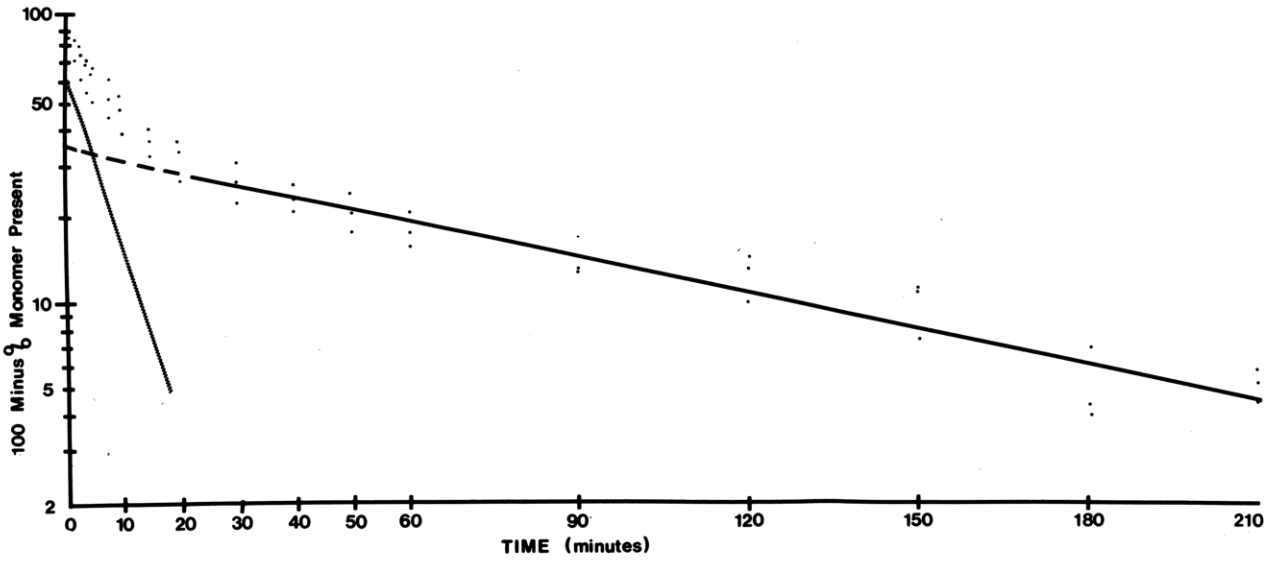

FIGURE 4: Kinetics of the appearance of M in semilog form. Results from three experiments are shown. The curve was resolved by the technique of exponential peeling (Frost & Pearson, 1961) into a slow phase (—) and a fast phase (···).

3 did not show strictly linear curves for either the disappearance of T plus T′ or the appearance of M. However, the semilog plot for the appearance of M (Figure 4) could easily be resolved by the technique of exponential peeling (Frost & Pearson, 1961) into two curves with linear slopes that differ by an order of magnitude. Approximately 65% of M appeared with a pseudo-first-order rate constant of 1.6×10^{-4} s^{-1} ($t_{1/2}$ = 72 min). The magnitude of this rate constant was similar to that determined from a semilog plot for the disappearance of D, which was linear and had a negative slope after 20 min, a time at which little carboxyl propeptide was present. ($t_{1/2}$ for the disappearance of D was 96 min; the plot is not shown.)

The remainder of M appeared 10 times more rapidly, with a pseudo-first-order rate constant of 2.4×10^{-3} s^{-1} and a half-time of 4.8 min. The half-time of appearance for this population of M was the same as that observed for the disappearance of the carboxyl propeptide (T plus T′).

When 1.0 mM DTT was used, M appeared with two pseudo-first-order rate constants of 7.0×10^{-4} and 8.3×10^{-3} s^{-1}. At both 0.5 and 1.0 mM DTT, the portion of M which was associated with the disappearance of the carboxyl propeptide appeared 10 times more rapidly than that resulting from the disappearance of D. Further, in the presence of either 0.5 or 1.0 mM DTT, the amount of M associated with the faster of the two apparent first-order reactions was approximately one-third, and that associated with the slower reaction was two-thirds of the final amount of M.

Rationale for the Use of the TPE Buffer System. The TPE buffer was chosen after preliminary experiments in a Tris–KCl–EDTA buffer (Creighton, 1978) revealed a series of proteins which electrophoresed at positions intermediate between M and fully reduced C1 (data not shown). That pattern suggested that both intra- and intersubunit disulfides were reduced. To simplify a potentially complicated reaction, we preferred to initially monitor only intersubunit disulfide reduction. Consequently, a series of buffers containing either KCl, Na_2SO_4, $(NH_4)_2SO_4$, or K_2HPO_4 (at 0.25, 0.5, or 1.0 M) with either 0.01 or 0.1 M Tris-HCl, pH 8.2, and 5 mM EDTA were assessed. Only reduction in the TPE buffer, used subsequently, revealed a single monomeric protein species.

It was of interest to note that in all of the buffer systems examined, no more than seven bands migrated in the monomer region. One of these seven species was M. Two other bands migrated with fully reduced C1 and C2. Two other species

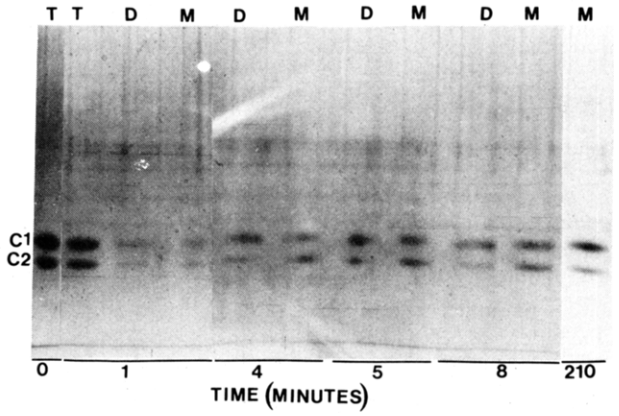

FIGURE 5: Electrophoretic separation of the components C1 and C2 of T plus T′, D, and M by reduction of disulfides in the presence of $NaDodSO_4$ as outlined in the text. Only T is indicated in the figure but includes any T′ that is present at 1 min. The times refer to time of alkylation during intersubunit disulfide reduction.

migrated slightly more rapidly than C1, and a final two, slightly more rapidly than C2. Migration through $NaDodSO_4$–polyacrylamide gels should be primarily, if not completely, dependent on the size and shape of a protein, and disulfide cross-links result in a more compact shape. Therefore, the $NaDodSO_4$ gel patterns after partial intrasubunit disulfide reduction in these different salt solutions suggested that both C1 and C2 have at least two intrasubunit disulfides.

Determination of C1 and C2 Content in D and M. In Figure 2, single dimeric and monomeric species are seen. It was unlikely that one of two potential dimers, $(C1)_2$ or (C1, C2), and one of two potential monomers, C1 or C2, were selectively precipitated during the reduction and therefore absent on electrophoresis since the quantity of stained material, as measured by densitometry, was constant within 10% over the course of the reaction. However, as only a single dimeric and monomeric species could be discerned on gels, it was not possible to directly determine the relative types of dimers and monomers existing at various times. Therefore, the bands corresponding to D and M were excised from the gels and electrophoresed into a second gel in the presence of DTT and $NaDodSO_4$ in order to reduce any existing disulfides.

Figure 5 shows a sample of D and M present at different times after complete reduction during the second electropho-

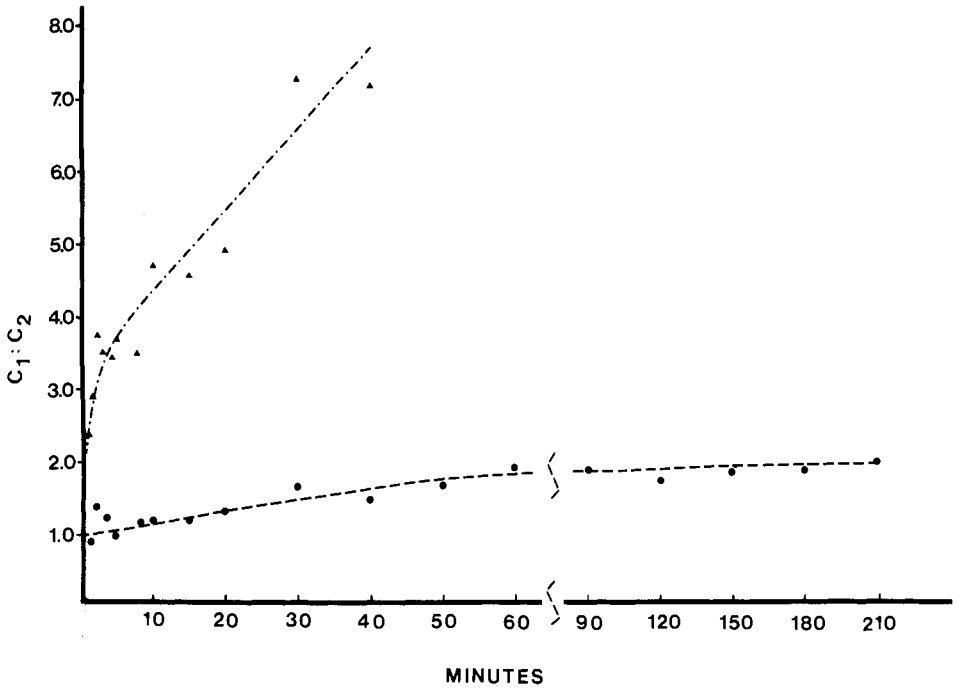

FIGURE 6: Ratio of the components, C1:C2, of D (▲) and M (●) trapped during the course of reduction. The ratios were determined from densitometry tracings of $NaDodSO_4$–polyacrylamide gels similar to those shown in Figure 5. Low quantities of D after 40 min precluded measurement of the C1:C2 ratio.

resis. When T was treated in this manner (Figure 5), densitometer tracings of the second electrophoresis revealed the expected 2:1 C1:C2 ratio. With increasing time of intersubunit disulfide reduction, the composition of M also approached and attained the expected 2:1 ratio (Figure 6). However, at early times, the ratio of C1 to C2 for M was approximately 1 to 1. The population of D, on the other hand, approached 4:1 at early times and contained increasing proportions of C1 at later times. This increase in C1 could be due to the intersubunit disulfides of the dimer, $(C1)_2$ being somewhat more resistant to reduction than those of the (C1, C2) dimer, or to an inability to measure the relatively smaller amounts of C2 that existed as the total amount of D decreased with time. Similar ratios of C1 to C2 for D and M at various times were found when 1.0 mM DTT was used for the reduction instead of 0.5 mM DTT.

The observed ratios suggested that reduction of the carboxyl propeptide resulted in approximately equal amounts of $(C1)_2$ and (C1, C2) with concomitant release of one C2 and one C1 molecule retaining intrasubunit disulfides, respectively. The ratios of C1 to C2 in Figure 6 were *not* those expected if an equal number of quasi-equivalent intersubunit disulfides were reduced by DTT in a random fashion. In that case, one would have expected that the ratio of C1 to C2 for both D and M would have been 2 to 1 at all times during the course of the reduction of the carboxyl propeptide.

Possible Model for the Pathway of Reduction of the Carboxyl Propeptide. The data presented show that the disappearance of the carboxyl propeptide proceeded via formation of a less compact trimer and then through dimers plus monomers, both of which retained intrasubunit disulfides. Reduction of intersubunit disulfides of these dimers followed at an overall rate which was approximately one-tenth of that at which the propeptide disappeared. Intrasubunit disulfides were also retained at this stage of reduction. It was impossible to distinguish between monomers by using gel electrophoresis until after all intrasubunit disulfides had been reduced.

The kinetics of reduction of the protein disulfides were expected to be pseudo first order with respect to protein concentration (Creighton, 1978). Within the limits of the techniques employed, two pseudo-first-order reactions could be discriminated. One reaction corresponded to the disappearance of T plus T′ with the concomitant appearance of one-third the final amount of M. The disappearance of T plus T′ and the appearance of this population of M had similar half-times. The second reaction corresponded to the disappearance of D with the concomitant appearance of two-thirds of the final amount of M. The rate of disappearance of D and its associated half-time could be estimated at times when the amount of D formed from T plus T′ was small in comparison to the amount of D present. These two pseudo-first-order reactions, the relative magnitudes of the half-times, and the relative amounts of M formed were observed whether 0.5 or 1.0 mM DTT was used in the reduction.

The methods used did not distinguish between the rates at which $(C1)_2$ and (C1, C2) appeared and disappeared. Therefore, the reactions

$$(C1_2, C2) \rightarrow (C1)_2 + C2$$

$$(C1_2, C2) \rightarrow (C1, C2) + C1$$

or the reactions

$$(C1)_2 \rightarrow 2C1$$

$$(C1, C2) \rightarrow C1 + C2$$

most likely occurred at similar rates, which suggests that one set of C1–C1 and C1–C2 intersubunit disulfide sites is similar.

Olsen et al. (1977) suggested that the carboxyl propeptide has approximately equal numbers of intra- and intersubunit disulfides. Data referred to above support this view, suggesting that each polypeptide chain has two intrasubunit disulfides for a total of six. With 23 cysteines (Fuller & Boedkter, 1981), there could be at most five intersubunit disulfides, which is roughly half the total possible number. The invariance in the positions of the cysteines in C1 and C2 suggests that the disulfides, whether intra- or intersubunit, will be homologous. The pattern shown at the top of Figure 7 seems realistic, assuming six intrasubunit and five intersubunit disulfides. The

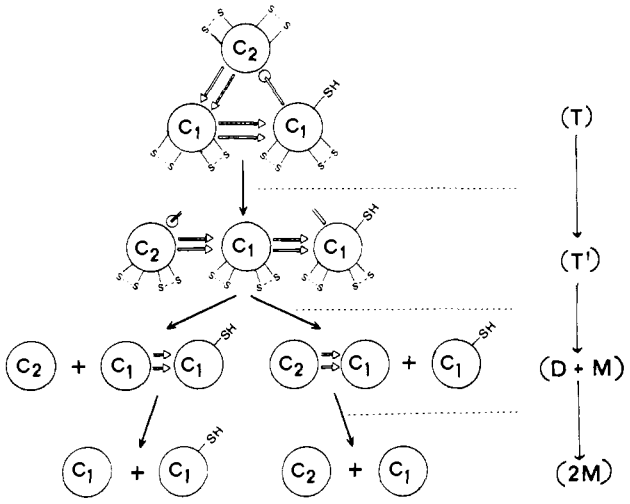

FIGURE 7: Schematic model for the intra- and intersubunit disulfide bond pattern of the carboxyl propeptide (T) is shown at the top of the figure, and the proposed scheme for disulfide reduction follows. Homologous intra- and intersubunit disulfides are indicated by the same symbols. The single intersubunit disulfide connecting the C1 and C2 polypeptides would be homologous with one of the two disulfides connecting the two C1 polypeptides and the other C1–C2 contact. Note that one of the C1 chains would have a free thiol. Formation of T′ as shown, with two sets of homologous disulfides, leads directly to the observed ratio of C1:C2 for D and M at early times. Although two intersubunit disulfides for each set of subunit contacts are shown for T′ and the dimers, strictly only a single intersubunit disulfide connecting each chain would be necessary. Intrasubunit disulfides and reduced intersubunit disulfides are omitted at the level of D and M in this figure only for the purpose of simplicity.

intersubunit surface contacts would be heterologous as expected for a trimer (Monod et al., 1965), but only two of the three sites would be strictly related. This pattern of intersubunit disulfides leads directly to the results of reduction reported within and to the hypothetical scheme for reduction shown in Figure 7. This pathway involves a minimum number of intermediates of disassociation.

If the single intersubunit disulfide, as proposed in Figure 7, were first reduced because it was more accessible to DTT, not only would a less compact trimer result, i.e., T′, but also this would yield a species where the remaining intersubunit disulfides were quasi-equivalent. Random reduction of intersubunit disulfides of T′ would then result in the experimentally determined proportions of $(C1)_2$ and (C1, C2). On the other hand, the species C1–C2–C1, or the species with all interchain disulfides and surrounding surfaces strictly related, would not result in the observed dimer pattern. However, if by some mechanism initial reduction of T to T′ was to result in equal amounts of C1–C2–C1 and C2–C1–C1, then by proposing that the C2–C1 disulfide linkage(s) was (were) more susceptible to reduction than the C1–C1 bond(s), one might also obtain the electrophoretic data seen. This might occur, for example, if the single C2–C1 disulfide schematically illustrated in Figure 7 is significantly more resistant to reduction than the other pairs of disulfide bonds between C1 and C2, and C1 and C1. Our data do not rule this possibility out, but it does seem unlikely that a pair of disulfides would be more readily cleaved than a single bond. Additional support for the T′ as a trimer where one intersubunit contact had been broken is noted in the fact that we have never observed a species which migrated more rapidly than M. Such a species could be expected if it were more highly cross-linked than M.

The approximately equivalent rates of dimer reduction also suggest that the C1–C1 and C1–C2 contact sites as indicated in Figure 7 are similar. But the much slower rate of disappearance of the dimers in comparison to the propeptide suggests that upon dimer formation, there had been conformational rearrangements leading to decreased accessibility of DTT.

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References

Bächinger, H. P., Bruckner, P., Timpl, R., Prockop, D. J., & Engel, J. (1980) *Eur. J. Biochem. 106*, 619.
Becker, U., Timpl, R., Helle, O., & Prockop, D. J. (1976) *Biochemistry 15*, 2853.
Bencze, W. L., & Schmid, K. (1957) *Anal. Chem. 29*, 1193.
Clark, C. C. (1979) *J. Biol. Chem. 254*, 10, 798.
Creighton, T. E. (1978) *Prog. Biophys. Mol. Biol. 33*, 231.
Crouch, E., & Bornstein, P. (1978) *Biochemistry 17*, 5499.
Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70.
Engel, J., Bruckner, P., Becker, U., Timpl, R., & Rutschmann, B. (1977) *Biochemistry 16*, 4026.
Frost, A. A., & Pearson, R. G. (1961) *Kinetics and Mechanism*, 2nd ed., pp 162–164, Wiley, New York.
Fuller, F., & Boedtker, H. (1981) *Biochemistry 20*, 996.
Gerard, S., Puett, D., & Mitchell, W. M. (1981) *Biochemistry 20*, 1857.
Harwood, R., Grant, M. E., & Jackson, D. S. (1976) *Biochem. J. 156*, 81.
Harwood, R., Merry, A. H., Woolley, D. E., Grant, M. E., & Jackson, D. S. (1977) *Biochem. J. 161*, 405.
Jimenez, S. A., Bashey, R. I., Benditt, M., & Yankowski, R. (1977) *Biochem. Biophys. Res. Commun. 78*, 1354.
Kao, W. W.-Y., Prockop, D. J., & Berg, R. A. (1979) *J. Biol. Chem. 254*, 2234.
Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Little, C. D., Church, R. L., Miller, R. A., & Ruddle, F. H. (1977) *Cell (Cambridge, Mass.) 10*, 287.
Mayne, R., Vail, M. S., Mayne, P. M., & Miller, E. J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1674.
Monod, J., Wyman, J., & Changeux, J.-P. (1965) *J. Mol. Biol. 12*, 88.
Murphy, W. H., von der Mark, K., McEneany, L. S. G., & Bornstein, P. (1975) *Biochemistry 14*, 3243.
Olsen, B. R., Guzman, N. A., Engel, J., Condit, C., & Aase, S. (1977) *Biochemistry 16*, 3030.
Pesciotta, D. M., Dickson, L. A., Showalter, A. M., Eikenberry, E. F., De Crombrugghe, B., Fietzek, P. P., & Olsen, B. R. (1981) *FEBS Lett. 125*, 170.
Rosenbloom, J., Endo, R., & Harsch, M. (1976) *J. Biol. Chem. 251*, 2070.
Saxena, V. P., & Wetlaufer, D. B. (1970) *Biochemistry 9*, 5015.
Schofield, J. D., Uitto, J., & Prockop, D. J. (1974) *Biochemistry 13*, 1801.
Smith, B. D., & Niles, R. (1980) *Biochemistry 19*, 1820.
Smith, B. D., Byers, P. H., & Martin, G. R. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3260.
Wohllebe, M., & Carmichael, D. J. (1978) *Eur. J. Biochem. 92*, 183.