Characterization of a Phosphorylated Form of the Intermediate Filament-Aggregating Protein Filaggrin†

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ABSTRACT: Filaggrin and a phosphorylated form of filaggrin, which has been shown by pulse–chase studies to be a precursor form of the protein [Dale, B. A., & Ling, S. Y. (1979) *Biochemistry 18*, 3539–3546], were compared for functional, biochemical, and physical properties. Filaggrin reacts with keratin filaments to form visible macrofibrils, unlike the precursor which does not. Biochemical and peptide-mapping studies suggest that the two proteins have similar, perhaps identical, amino acid sequences. The major differences between the two proteins are in molecular weight (precursor, 44 200 g/mol; filaggrin, 38 400 g/mol), the existence of oligomeric forms of the precursor, and the presence of phosphate in the precursor (15–20 mol/mol of protein). Phosphoserine was identified in the precursor, but neither phosphothreonine nor phosphotyrosine was observed. The results of proteolytic digests of [^{32}P]phosphate-radiolabeled precursor show that the phosphate is unevenly distributed throughout the molecule and may be localized in approximately 30% of the precursor. A discrete localization of the phosphate in the precursor may block a specific keratin filament combining site and so prevent premature aggregation of these filaments during epidermal differentiation. It is suggested that a specific phosphatase is involved in the dephosphorylation, because several phosphatases of general specificity, including rat epidermal lysosomal acid phosphatase, did not catalyze this conversion.

A major function of skin is the protection of the soft underlying tissues from physical, chemical, and microbial attack. This protection is accomplished by the organization of the outermost layer of the skin (the stratum corneum), which consists of stacked interdigitating cells that have thickened cell membranes and are devoid of nuclei and other organelles. When stained with osmium tetraoxide, the interior of a stratum corneum cell shows a pattern of lightly stained keratin filaments embedded in a darkly stained matrix. The staining characteristics of this interfilamentous matrix are similar to those of the amorphous, amembranous keratohyalin granules found in the living cells immediately adjacent to the stratum corneum. This led Brody (1959) to propose that the interfilamentous matrix is derived from the keratohyalin granules in the adjacent cell layer. Recent biochemical evidence, described below, supports this hypothesis.

In vitro, a basic (cationic) protein from the stratum corneum (Dale, 1977) was shown to interact specifically with intermediate filaments, such as keratin, to form tightly aligned macrofibrils whose ultrastructure resembles that of the keratin filaments in the stratum corneum (Dale et al., 1978, 1981; Steinert et al., 1981). This basic protein is therefore presumed to be the interfilamentous matrix protein. It is now called filaggrin (Steinert et al., 1981). Immunologic and pulse–chase studies indicate that filaggrin is dervied from a precursor found in the keratohyalin granules (Ball et al., 1978; Dale & Ling, 1979a,b). We have purified this precursor and have compared it with filaggrin. The results are consistent with a close relationship between the two proteins, although unlike filaggrin, the precursor is a phosphorylated protein (Lonsdale-Eccles et al., 1980).

Filaggrin, which has also been called stratum corneum basic protein, histidine-rich protein II, and histidine-rich basic protein, is a member of a group of histidine-rich proteins found in mammalian keratinizing epithelia such as epidermis (Ugel, 1969; Balmain et al., 1977; Ball et al., 1978; Dale & Ling, 1979a,b; Dale et al., 1982a,b; Lonsdale-Eccles et al., 1981; Scott & Harding, 1981). In addition to histidine, these proteins are rich in arginine, glutamic acid, serine, glycine, and alanine, but they are deficient in hydrophobic amino acids including methionine. The lack of methionine has been used to detect the proteins in a variety of epithelia (Dale et al., 1981; Lonsdale-Eccles et al., 1981). In addition to their similar amino acid compositions, the filaggrins or histidine-rich proteins exhibit immunologic cross-reactivity between species [rat/human, Lynley & Dale (1982) and A. M. Lynley and B. A. Dale (unpublished experiments); rat/mouse, Holbrook et al. (1982) and Steinert et al. (1981)]. Despite these similarities, the proteins are species distinct as shown by their different molecular weights and their slightly different amino acid compositions. Thus, these proteins are members of a family of biochemically related, yet species-specific, proteins that are intimately involved in the terminal differentiation of keratinization. In this report we give a detailed description of the filaggrin precursor from newborn rat, compare it with filaggrin, and discuss some problems involved in converting one into the other.

Experimental Procedures

Materials. Ampholytes and Bio-Gel P-2 (100–200 mesh) were obtained from Bio-Rad Laboratories, Richmond, CA; Sephacryl S-200 superfine and S-300 superfine were from Pharmacia Fine Chemicals, Piscataway, NJ; acid phosphatase (0.158 unit/mg; catalog no. 1161 AP), alkaline phosphatase (48 units/mg; no. 6125 BAPF), elastase (62 units/mg; no. 6363 ESFF), carboxypeptidase A (10 mg/mL), and pyruvate kinase (no. 3279 PKL) were from Worthington Biochemical Corp., Bedford, MA; endoproteinase Lys-C was from Boehringer Mannheim Biochemicals, Indianapolis, IN; *Staphylococcus aureus* V8 protease was a gift from K. Titani, Univ-

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ersity of Washington; other proteases and proteins were from Sigma Chemical Co., Saint Louis, MO; radiolabeled proteins (for sodium dodecyl sulfate ($NaDodSO_4$)[1]–polyacrylamide gel calibration) were from Bethesda Research Laboratories, Bethesda, MD; sodium [^{32}P]phosphate, [^{14}C]- and [^{3}H]-histidine, Aquasol-2, and En^{3}hance (used for fluorography of $NaDodSO_4$–polyacrylamide gels as described by the manufacturer) were from New England Nuclear, Boston, MA. Eagle's culture medium was from Gibco Laboratories, Grand Island, NY; guanidine hydrochloride, was from Heico Inc., Delaware, NJ.

Preparation of Filaggrin and Its Precursor. The filaggrin precursor was prepared as described by Lonsdale-Eccles et al. (1980), except that the 1 M potassium phosphate extract was made 9% in formic acid, and centrifuged to remove the precipitate, prior to concentration on an Amicon PM10 ultrafiltration membrane. Newborn rat filaggrin was prepared as described by Dale (1977). For sedimentation equilibration analysis, the rat filaggrin (20 mg) was further purified by $NaDodSO_4$–polyacrylamide gel electrophoresis on a Buchler preparative column, no. 200, by using the Laemmli (1970) discontinuous buffer system with 90 mL of 10% resolving gel and 20 mL of 3.3% stacking gel. The protein was identified by its relatively high absorbance at 235 vs. 280 nm and by analytical $NaDodSO_4$–polyacrylamide gel electrophoresis. The peak containing filaggrin was dialyzed against H_2O and lyophilized, and the $NaDodSO_4$ was removed by ion-pair extraction (Henderson et al., 1979). Less than 1% $NaDodSO_4$ remained after the extraction (Waite & Wang, 1976).

The high molecular weight form of the precursor was extracted from newborn rat epidermis with 8 M urea–0.05 M Tris-HCl, pH 7.4.

Incorporation of [^{32}P]Phosphate. Newborn rats were injected subcutaneously at the start of a 3-h labeling period with 0.15 M NaCl containing [^{32}P]phosphate (50, 100, and 400 μCi/animal). For time-course studies a constant amount of [^{32}P]phosphate (150 μCi/animal) was injected, and groups of rats were killed at 0.5, 1.5, 3, and 5 h after injection. The epidermis was obtained and extracted with 1 M potassium phosphate (Lonsdale-Eccles et al., 1980). The potassium phosphate extracts were dialyzed against distilled water and centrifuged at 40000g for 40 min. The pellets were dissolved in a small volume of 8 M urea with 0.05 M Tris-HCl (pH 7.3) and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis and fluorography. For the phosphate-characterization studies, the labeled precursor was further purified as previously described (Lonsdale-Eccles et al., 1980).

For the incorporation of [^{32}P]phosphate in organ culture, dorsal skins of five newborn rats were removed and then incubated for 3 h at 37 °C in 3 mL each of minimum Eagle's medium containing Earle's salts, glutamine (0.3 g/L), and 140 μCi of sodium [^{32}P]phosphate, after which the epidermis was separated from the dermis by the EDTA method (Holbrook et al., 1982). The labeled precursor was extracted with a combination of 1 M potassium phosphate (containing histidine and phenylmethanesulfonyl fluoride) and 8 M urea (containing 2% β-mercaptoethanol) and purified as above.

Two-Dimensional Analysis. Isoelectric focusing was performed overnight (>18 h) in polyacrylamide tube gels as described by Miner & Heston (1972) with the modifications of Wrigley (1971). The ampholyte was a mixture of two parts pH 8–10 and eight parts pH 3–10, and the gels were fixed and stained by using the Jackle (1979) procedure. Electrophoresis in the second dimension was performed on a $NaDodSO_4$–7.5% polyacrylamide gel. The pH profile of the isoelectrically focused gel was obtained from a tube gel focused in parallel with the sample.

Peptide Mapping. [^{32}P]Phosphate-labeled precursor was digested with chymotrypsin and with endoproteinase Lys-C. (a) Chymotryptic digestions of in vivo labeled precursor (1.5 mg/mL in 50 mM Tris-HCl) were performed at pH 8.8, 37 °C, by using 12 μg/mL enzyme and also at pH 6.8 by using 120 μg/mL enzyme. (b) Chymotryptic digestion of precursor labeled in vitro was performed in 0.2% $NaDodSO_4$, 3 M urea, and 300 mM Tris-HCl, pH 6.8, with 70 μg/mL chymotrypsin. (c) Endoproteinase Lys-C digestion of precursor (6 mg/mL) radiolabeled in vivo was performed in 40 mM Tris-HCl, pH 7.7, 3.3 M urea, 0.4 mM glycylglycine, and 0.7 mM EDTA with 8 μg/mL enzyme. Aliquots were removed at timed intervals, boiled for 3 min to terminate the reaction, and stored at −20 °C until required for analysis by $NaDodSO_4$–polyacrylamide gel electrophoresis on gradient gels.

One-dimensional peptide maps of [^{14}C]histidine-labeled filaggrin and precursor were obtained by the method of Cleveland et al. (1977). The digestions were carried out for 30 min with elastase (1 ng–10 μg), 15 min with ficin (0.05–500 ng), and 45 min with *S. aureus* V8 protease (0.05–500 ng). The protein concentrations of filaggrin and its precursor were approximately equal (10–20 μg/lane), but the specific radioactivity of the precursor was about 3-fold higher than that of the filaggrin. The exposure time during fluorography (Bonner & Laskey, 1974) was adjusted accordingly.

Molecular Weight Determinations. Solutions of filaggrin or monomeric precursor (1 mg/mL in guanidine hydrochloride) were dialyzed against 100 mL of 6 M guanidine hydrochloride for 48 h and then examined by analytical ultracentrifugation as described for the determination of mouse filaggrin (Steinert et al., 1981). Molecular weights were computed as described by Teller (1973). Values for ϕ', the apparent specific volume, in 6 M guanidine hydrochloride were calculated by the method of Lee & Timasheff (1974). The phosphate in the protein was accounted for as seryl phosphate, the specific volume of which was determined by McMeekin et al. (1949). The values of ϕ' from the composition were 0.666 mL/g for the precursor and 0.672 mL/g for filaggrin. With the density of 6 M guanidine hydrochloride taken as 1.1418 g/mL (Lee & Timasheff, 1972), these values give $(\partial\rho/\partial c)_\mu = 0.2400$ and 0.2323 for the precursor and filaggrin, respectively.

Analytical $NaDodSO_4$–polyacrylamide gel electrophoresis was done on 1.5-mm slab gels as described by Laemmli (1970). Acrylamide concentrations were varied between 5% and 15% w/v. Ferguson (1964) plots were constructed from the R_f values obtained from these gels. Phosphorylase b (M_r 93 000), bovine serum albumin (M_r 67 000), pyruvate kinase (M_r 57 000), ovalbumin (43 000), and chymotrypsinogen (M_r 25 000) were used as standards.

Macrofibril Formation. Newborn rat keratin filaments were extracted and re-formed (×2) by dialysis against 5 mM Tris-HCl, pH 7.6, containing 10 mM β-mercaptoethanol and 1 mM EDTA (Steinert et al., 1976). $NaDodSO_4$ gels of this preparation showed the presence of keratin proteins only and no contaminating filaggrin. Proteins to be combined with the filaments were dissolved in the same buffer. Rat filaggrin or its precursor was mixed with keratin filaments at a concentration of approximately 300 μg/mL filament protein, negatively stained with 1% aqueous uranyl acetate, and examined

[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

by transmission electron microscopy on parlodion and carbon-coated grids.

Chemical Characterization. The free carboxyl groups of filaggrin and the precursor were modified by reacting 0.5 mg of the proteins with 0.5 mg of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride in 50 μL of 7 M ethylamine–6 M guanidine hydrochloride–0.1 M quinoline (pH 4.75). After 18 h the reaction was terminated by separating the reactants on a Bio-Gel P-2 column (10 × 0.7 cm) preequilibrated in 10 mM HCl. The proteins were lyophylized, hydrolyzed in 5.7 N HCl at 110 °C in vacuo for 18 h, and then subjected to amino acid analysis.

Cyanogen bromide cleavage of proteins was performed as described by Dale & Ling (1979a). Hydroxylamine cleavage was performed on the high molecular weight species of precursor by a minor modification of the method described by Lam & Kasper (1980).

Automated sequence analysis of the amino terminus of filaggrin and the precursor was performed on the Beckman sequencer, Model 890 B, by the method of Edman & Begg (1967) as modified by Hermodson et al. (1972).

Carboxy-terminal analysis was performed by incubating 10 mg/mL of filaggrin or the precursor with a mixture of equal amounts of carboxypeptidases A and B (enzyme:substrate ratio of 1:25) in 0.2 M *N*-ethylmorpholine acetate (pH 8.5)–6 M urea. At selected time intervals, 0.5-mg aliquots were removed, acidified with 9% formic acid, and lyophilized. The samples were analyzed on a Beckman 121 amino acid analyzer that had been modified for microanalytic use.

Phosphate and Hexose Analyses. Phosphate analysis was performed by the method of Sumner (1944) as modified by Bitte & Kabat (1974). Phosphoserine was identified by high-voltage paper electrophoresis after hydrolysis in 5.7 N HCl at 110 °C. Hexosamine was assayed by the method of Gatt & Berman (1966), and total hexose was assayed by the method of Scott & Melvin (1953). Approximately 0.5 mg of protein was used in each analysis. Filaggrin and the precursor were also analyzed for carbohydrate content by gas chromatography/mass spectral analysis.

Acid and Alkaline Phosphatase. [^{14}C]Histidine-labeled filaggrin precursor was dissolved in 150 μL of 8 M urea (1 mg/mL) and then dialyzed against 500 mL of 0.5 M Tris-HCl, pH 7.6, or 0.25 M sodium acetate, pH 5.0, for 1 h. Alkaline phosphatase (50 units/mg) and acid phosphatase (0.16 unit/mg) were dissolved in the pH 7.6 and pH 5.0 buffers, respectively. The dialyzed precursor samples were treated separately with the respective enzyme solutions for 1 h at 37 °C by using enzyme:substrate ratios ranging from 1:5 to 1:50 000. After the incubation, the samples were analyzed by electrophoresis in $NaDodSO_4$–polyacrylamide gels.

Rat Acid Phosphatase. This enzyme was extracted and purified from newborn rat epidermis as described for the preparation of guinea pig epidermal acid phosphatase by Miyagawa et al. (1977) except that Sephacryl S-200 was used in place of Sephadex G-100 as the gel filtration medium. The enzyme was eluted from a CM-cellulose column between 0.12 and 0.14 M NaCl with a linear salt gradient ranging from 0 to 0.4 M NaCl in 150 mL of the acetate buffer. Column fractions (0.1-mL aliquots) were assayed for enzyme activity as described by Miyagawa et al. (1977). One unit of enzyme is defined as that amount which catalyzes the hydrolysis of 1 nmol of *p*-nitrophenyl phosphate in 1 min under the standard assay conditions.

Aliquots of this purified acid phosphatase (2 × 150 units added at 0 and 3 h) were added to [^{32}P]phosphate-labeled precursor and incubated in 0.1 M sodium acetate, pH 5.0, at 37 °C for a total of 20 h. A similar sample was pretreated with trypsin (2 × 5 μg of trypsin added to 250 μg of precursor in 0.1 M ammonium bicarbonate at 0 and 3 h; reaction terminated at 4.5 h by the addition of 10% formic acid and lyophilization) and then incubated with the phosphatase as described above. Each sample was chromatographed on a Bio-Gel P-2 column (24.5 × 0.9 cm) in 1 mM HCl. The percentages of radiolabel in the peaks corresponding to the void volume, the inorganic phosphate elution volume, and the separation volume were calculated.

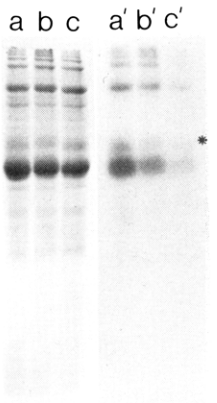

FIGURE 1: Dose-dependent incorporation of [^{32}P]phosphate into filaggrin precursor. [^{32}P]Phosphate [400 μCi (a), 100 μCi (b), and 50 μCi (c)] was injected subcutaneously into newborn rats; after 3 h the rats were killed, the precursor was isolated, and the protein extracts were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis as described in the text. The Coomassie brilliant blue stained gels are lanes a–c, and the radioautographs are lanes a′–c′. The asterisk indicates the protein that is cleaved in the presence of cyanogen bromide.

Results

Radioactive Phosphate Incorporation. The precursor of filaggrin has been shown to contain 15–20 mol of phosphate/mol of protein (Lonsdale-Eccles et al., 1980). After partial acid hydrolysis, a spot comigrating with standard phosphoserine is observed in the precursor preparation (data not shown). Phosphothreonine and phosphotyrosine, which comigrate in the system used (Hunter & Sefton, 1980), are not observed. This precursor can be labeled in vivo with [^{32}P]phosphate by subcutaneous injection of labeled phosphate and in vitro by organ culture of skin in the presence of labeled phosphate. The [^{32}P]phosphate incorporation in vivo exhibits both dose (Figure 1) and time dependence (not shown). Phosphate is incorporated into the M_r 54 000 precursor and into a number of higher molecular weight bands that form a polymeric series. As shown below, the M_r 54 000 band is the lowest member of the series. The label is also incorporated into an unrelated protein of molecular weight slightly higher than that of the monomeric precursor (Figure 1). Unlike the proteins in the polymeric series, this protein is not labeled with [^{3}H] histidine and is unstable in the presence of cyanogen bromide (data not shown). Thus, this protein is unrelated to the filaggrins.

Two-dimensional electrophoresis (isoelectric focusing followed by $NaDodSO_4$–polyacrylamide gel electrophoresis) of the monomeric and polymeric species of the precursor shows them to be neutral molecules (Figure 2). The monomer, with a p*I* in the range 6.9–7.1, shows slight heterogeneity in the higher pH range that may be a consequence of incomplete phosphorylation of these molecules. The p*I* range of the polymeric species is slightly lower than that of the monomer and may indicate that the polymers are more phosphorylated

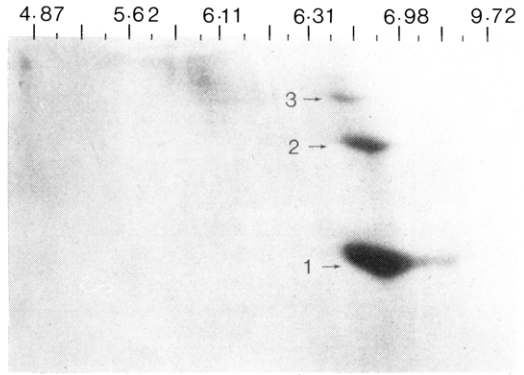

FIGURE 2: Fluorograph of [^{32}P]phosphate-labeled precursor after two-dimensional electrophoresis. Isoelectric focusing (pH 3–10) was performed in the first dimension and NaDodSO$_4$–polyacrylamide gel electrophoresis in the second dimension, as described under Experimental Procedures. The Coomassie brilliant blue stained gel was identical with the fluorograph shown. The monomer, dimer, and trimer are indicated by 1, 2, and 3, respectively.

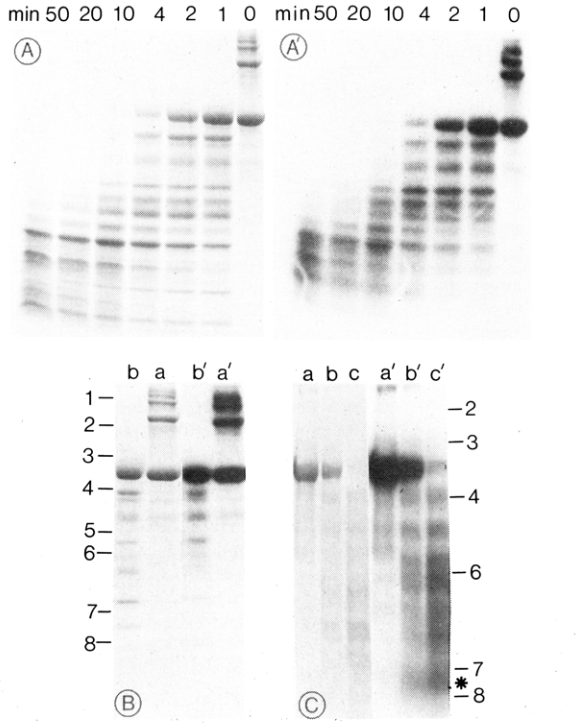

FIGURE 3: NaDodSO$_4$–polyacrylamide gel electrophoresis of [^{32}P]-phosphate-labeled precursor after digestion with chymotrypsin. (A) Precursor labeled in vivo was digested by chymotrypsin at pH 6.8 with 120 μg/mL enzyme; (A) is the Coomassie brilliant blue stained gel and (A′) the fluorograph. The digest was performed as described under Experimental Procedures, aliquots were removed at the times indicated above each lane (minutes), and the reaction in each aliquot was terminated by boiling for 3 min. (B) Precursor labeled in vivo was digested at pH 8.8 with 12 μg/mL enzyme. (C) Precursor labeled in vitro was digested with 70 μg/mL chymotrypsin, pH 6.8, in the presence of 0.2% NaDodSO$_4$ and 3 M urea. The analyses were performed on polyacrylamide gradient gels [10–20% (A and B); 10–15% (C)]. The Coomassie brilliant blue stained gels of (B) and (C) are indicated by (a–c) and the fluorographs by (a′–c′). The lanes a, b, and c are the 0-, 1-, and 3-min aliquots. The standards used were (1) myosin (M_r 200 000), (2) phosphorylase *b* (M_r 93 000), (3) bovine serum albumin (M_r 68 000), (4) ovalbumin (M_r 43 000), (5) deoxyribonuclease I (M_r 31 000), (6) chymotrypsin (M_r 25 000), (7) β-lactoglobulin (M_r 18 400), (8) cytochrome *c* (M_r 12 300), and (9) aprotinin (M_r 6500). (A) and (B) are photographs of different portions of the same slab gel. The asterisk marks the highly labeled (with [^{32}P]phosphate) peptide that is associated with minimal protein staining.

than the monomer, although no such difference was discernible by colorimetric assay for phosphate. The slight streaklike

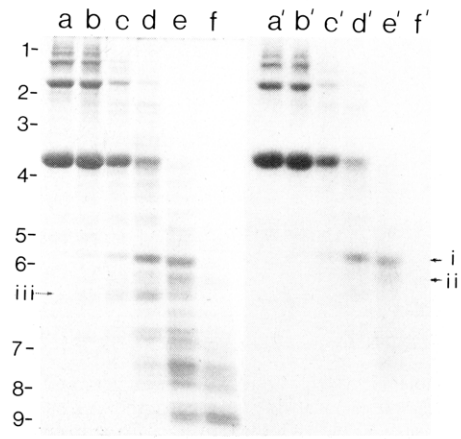

FIGURE 4: NaDodSO$_4$–polyacrylamide gel electrophoresis of [^{32}P]-phosphate-labeled precursor after digestion with endoproteinase Lys-C. Endoproteinase Lys-C digest of precursor labeled in vivo was performed at pH 7.7 as described under Experimental Procedures. Aliquots were removed at (a) 0 min, (b) 10 min, (c) 30 min, (d) 2 h, (e) 6 h, and (f) 20 h, boiled, and then subjected to electrophoresis on a 10–15% polyacrylamide gel by using the standards described in Figure 3. The Coomassie brilliant blue stained gel is indicated by (a–f) and the fluorography by (a′–f′). The radiolabeled peptides described in the text (M_r 30 000 and M_r 25 000) are indicated by arrows (i and ii) and the M_r 23 000 band is indicated by (iii).

appearance of the proteins may be a consequence of the relative insolubility of the precursor proteins at neutral pH. The unrelated phosphorylated protein seen in Figure 1 is not observed in this gel; it may have a p*I* lower than that resolved by this isoelectric focusing system.

Phosphate Localization. To determine whether the [^{32}P]-phosphate radioactivity is dispersed throughout the protein or localized in a specific region, the protein was digested with proteolytic enzymes and analyzed by NaDodSO$_4$–polyacrylamide gel electrophoresis and fluorography (Figures 3 and 4). The chymotryptic digest (Figure 3A) shows a large number of stained protein bands that have a corresponding radioactive band. However, the relative amount of radioactivity associated with each band is not constant, and a number of the smaller peptides appear to have no phosphate. Complementing these is a highly phosphorylated peptide of approximately M_r 15 000 (approximately 30% of precursor) that was identified in a chymotryptic digest of precursor [^{32}P]phosphate labeled in vitro (Figure 3C).

An uneven distribution of phosphate within the molecule is also shown by the endoproteinase Lys-C digestion of the precursor (Figure 4). This enzyme, which is reported by the manufacturer to be highly specific for lysine residues, initially breaks the precursor into two fragments of M_r 30 000 and M_r 23 000 (a diffuse band). The M_r 30 000 fragment is heavily phosphorylated, whereas the M_r 23 000 fragment is not. This unphosphorylated fragment corresponds to approximately 40% of the precursor. Both fragments are further broken down to small peptides, the M_r 30 000 via an M_r 25 000 intermediate that is also phosphorylated.

The endoproteinase Lys-C cleavage of the monomeric and oligomeric forms of the precursor proceeds at approximately the same rate. However, in the presence of chymotrypsin, the oligomeric forms are cleaved at a much faster rate than is the monomeric form (Figure 3A). Even when a 10-fold dilution of enzyme was employed in the digestion (Figure 3B) the oligomers were broken down within 1 min, although the monomer was still incompletely digested even after 50 min. In either case, the chymotryptic fragments produced by the almost instantaneous breakdown of the oligomeric forms do not

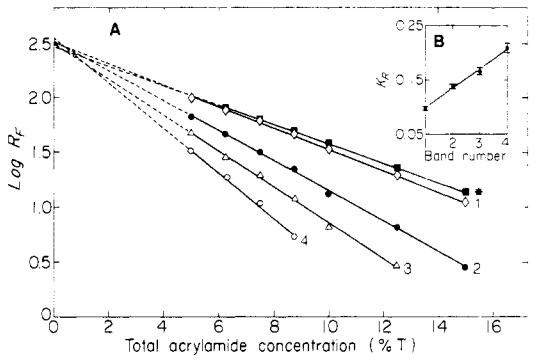

FIGURE 5: Molecular weight analysis of filaggrin and precursor(s) by $NaDodSO_4$–polyacrylamide gel electrophoresis as described by Ferguson (1964). (A) Plot of logarithm R_f of filaggrin and of precursor bands against acrylamide concentration. 1, 2, 3, and 4 refer to the monomer, dimer, trimer, and tetramer of the precursor, and the asterisk refers to filaggrin. (B) Plot of retardation coefficient, K_r (the slopes of lines 1–4), of precursor bands against the integral band number of precursor (from Figure 6A).

differ from those produced by the subsequent cleavage of the monomeric form, a result that supports the hypothesis that the monomeric and oligomeric forms are related proteins. Immunological (Dale & Ling, 1979b), molecular weight (Figure 7), and phosphate and histidine radiolabel incorporation data also support this hypothesis. However, the oligomeric forms must have a different conformation from that of the monomer that results in an increased exposure of the sites at which chymotrypsin cleaves the molecules. The sites in the monomer must be relatively masked.

The large number of peptides produced by both the chymotrypsin and the endoproteinase Lys-C digestions suggests that these enzymes may be contaminated with trace amounts of trypsin-like enzymes. To test this possibility, we repeated the above digestions in the presence of 0.5 mM *p*-aminobenzamidine (a potent trypsin inhibitor). No change in the cleavage pattern was observed, and so the cleavages are presumed to occur only at chymotryptic and endoproteinase Lys-C recognition sites. Endoproteinase Lys-C may have less affinity for arginine residues than for lysine residues rather than no affinity at all as claimed by the manufacturer.

Molecular Weight Determination. $NaDodSO_4$–polyacrylamide gel electrophoretic analysis of filaggrin and the monomeric precursor yields values of M_r 49 000 and M_r 54 000, respectively, a difference of 5000. However, Ferguson plots of the R_f values of filaggrin, the precursor proteins, and protein standards following electrophoresis in $NaDodSO_4$–polyacrylamide gels of different concentrations (Figure 5) show that the intercepts of the precursor bands (2.52 ± 0.02) differ from those of filaggrin and the standard proteins (2.46 ± 0.01). Thus either or both of the proteins migrate anomalously. Furthermore, compared with most globular proteins that bind approximately 1.4 mg of $NaDodSO_4$/mg of protein (Nielsen & Reynolds, 1978), filaggrin binds an unusually large amount of $NaDodSO_4$ (4.3 mg of $NaDodSO_4$/mg of filaggrin). This may account for the difference in migration between basic proteins and the commonly used standards and indicates that the molecular weights of filaggrin and the precursor, as measured by $NaDodSO_4$–polyacrylamide gel electrophoresis, are probably unreliable. To check this, we analyzed their molecular weights by sedimentation equilibrium analysis, a method that is based on totally different physical and theoretical parameters.

Sedimentation equilibrium analyses of filaggrin and the purified monomeric form of the precursor result in computed minimum molecular weights of 38 400 ± 400 and 44 300 ± 200, respectively (Figure 6). These values differ significantly from those calculated by $NaDodSO_4$–polyacrylamide gel electrophoresis and confirm the unreliability of the latter method.

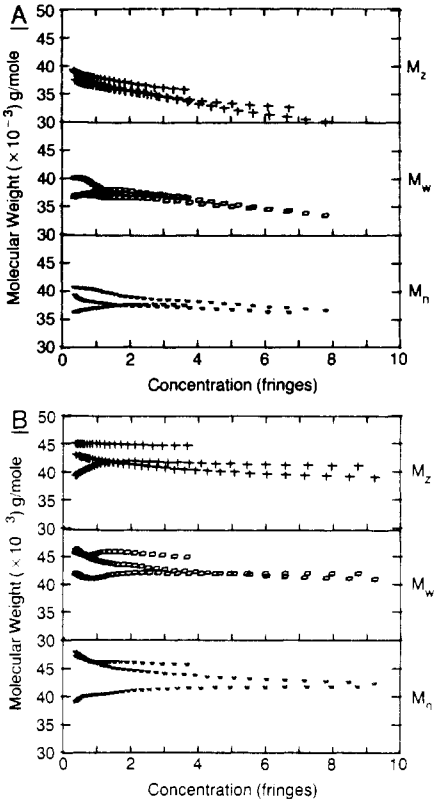

FIGURE 6: Molecular weight distribution of newborn rat filaggrin (A) and filaggrin precursor (B) obtained by equilibrium sedimentation. Data presented are computer-generated plots of point-by-point molecular weight average vs. concentration (fringes) from analysis of a Rayleigh plate. Initial loading concentrations of approximately 0.1, 0.67, and 0.33 mg/mL were centrifuged for 48 h at 32 000 rpm and 20 °C in 6 M guanidine hydrochloride. The following molecular weight averages were obtained by extrapolation of point-by-point values to $c = 0$. For filaggrin, M_1 = 38 400 ± 150, M_n = 38 300 ± 170, M_w = 38 600 ± 130, and M_z = 38 980 ± 150. For precursor, M_1 = 44 210 ± 190, M_n = 44 020 ± 430, M_w = 43 980 ± 290, and M_z = 44 390 ± 360. M_1 is the estimate of smallest molecular weight species computed from the point-by-point molecular weight averages and extrapolated to $c = 0$ (Teller, 1973). M_n, M_w, and M_z represent the number-average, the weight-average, and the z-average molecular weights, respectively, extrapolated to $c = 0$.

The sedimentation equilibrium plots of molecular weight of the precursor (Figure 6B) indicate that there is slight heterogeneity of this preparation. We therefore calculated the whole-cell average molecular weight data by the methods described by Harris et al. (1969) with the constraint that the slopes (virial coefficient) be the same for the molecular weight averages of each protein. Within experimental error, the virial coefficient for the precursor [(4.0 ± 0.5) × 10^{-4} mol g^{-1} $fringe^{-1}$] is the same as for filaggrin [(5.5 ± 1.4) × 10^{-4} mol g^{-1} $fringe^{-1}$]. In this method of calculation, after extrapolation to $c = 0$, a lowered estimate of the minimum molecular weight will be obtained if the preparation contains higher molecular weight species and vice versa. The recalculated value for the molecular weight of filaggrin (M_1 = 38 700 ± 100) is not significantly different from that obtained from the point-by-point average calculation (M_1 38 400 ± 150; Figure 6), indicating its homogeneity. The recalculated molecular weight (M_1 = 43 300 ± 200) of the precursor preparation is 900 g/mol lower than that obtained by the extrapolation to $c = 0$ from point-by-point molecular weight averages (M_1 = 44 210 ± 190;

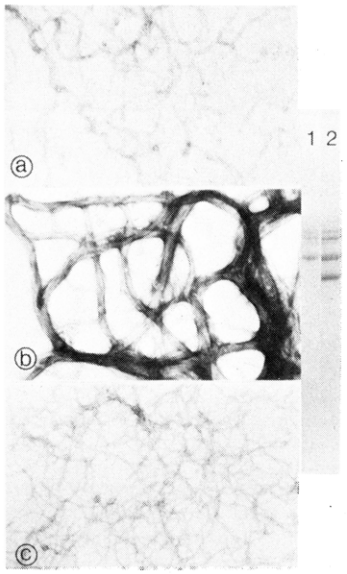

FIGURE 7: Keratin filament reaction with filaggrin and its precursor. (a) Keratin filaments alone; (b) keratin filaments and filaggrin; (c) keratin filaments and precursor. Equal proportions of each reactant were used in (b) and (c); 0.25 filaggrin (or precursor):1.0 keratin (w/w). The aggregation of keratin filaments into macrofibrils occurs only in the presence of filaggrin. Lane 1, the $NaDodSO_4$–polyacrylamide gel electrophoretic analysis of the rat keratin filament preparation, shows the absence of filaggrin in the filament preparation. Lane 2 is the keratin filament preparation plus filaggrin.

Figure 6). The two values are close and indicate that the degree of heterogeneity in the precursor preparation is small. Both methods of calculation clearly confirm the difference of approximately 5000 in the molecular weight of the two forms of the protein.

Physicochemical Properties. Reactivity with rat keratin filaments was tested for both filaggrin and the monomeric precursor. At equivalent protein ratios [0.25:1 precursor (or filaggrin):keratin filament protein (w/w)] reaction with filaggrin resulted in the aggregation of keratin filaments into macrofibrils. The precursor did not react to form macrofibrils (Figure 7).

Despite their differences in reactivity with keratin filaments, filaggrin and precursor have very similar amino acid compositions, each being deficient in hydrophobic residues and rich in polar residues (Table I). The ultraviolet spectra of filaggrin and precursor show little absorbance at 280 or 260 nm, which confirms the lack of hydrophobic amino acids in the proteins and the absence of nucleic acids in the phosphate-containing precursor. At protein concentrations of 1 mg/mL (in 6 M urea–0.02 M Tris-HCl (pH 7.6)–1 mM glycylglycine), the precursor and filaggrin had 0.15 *A* at 280 nm, 0.16 *A* at 260 nm, and 1.0 *A* at 235 nm. Titration of the free carboxyl groups with ethylamine shows that both proteins have identical amounts (50%) of their glutamic and aspartic acids in the free acid form and the remainder in the presumed amide form, asparagine or glutamine (Table I).

Digestion of filaggrin and the precursor with a mixture of carboxypeptidases A and B resulted in the release of only arginine from both proteins. Thus, both proteins have C-terminal arginine residues, a result that is consistent with that described by Bernstein (1970) for the histidine-rich proteins. Automated Edman degradation of the proteins failed to release any amino acids, which suggests that they have blocked N-terminal amino acids.

The marked similarities between the proteins were confirmed by peptide-mapping studies (Figure 8). Elastase and

Table I: Amino Acid Analysis of Ethylamine-Treated Filaggrin and Precursor[a]

	filaggrin	precursor
Asx	4.3	5.1
Glx	17.8	19.1
Asp + Glu	11.2	11.9[b]
Thr	6.3	5.9
Ser	16.1	14.1
Pro	3.4	3.3
Gly	15.7	13.5
Ala	11.5	11.1
Val	0	1.1
Met	0	0.3
Ile	2.2	2.3
Leu	0.8	1.4
Tyr	0.2	0.5
Phe	0	0.6
His	7.0	7.1
Lys	1.0	1.6
Arg	14.1	13.4

[a] The compositions are expressed as residues per 100 and are the averages of two independent analyses. [b] The aspartic and glutamic acid content is obtained from the γ-(ethylamido)-glutamyl (or aspartyl) residues prepared by coupling ethylamine to the free carboxyl groups of the proteins with water-soluble carbodiimide, as described in the text.

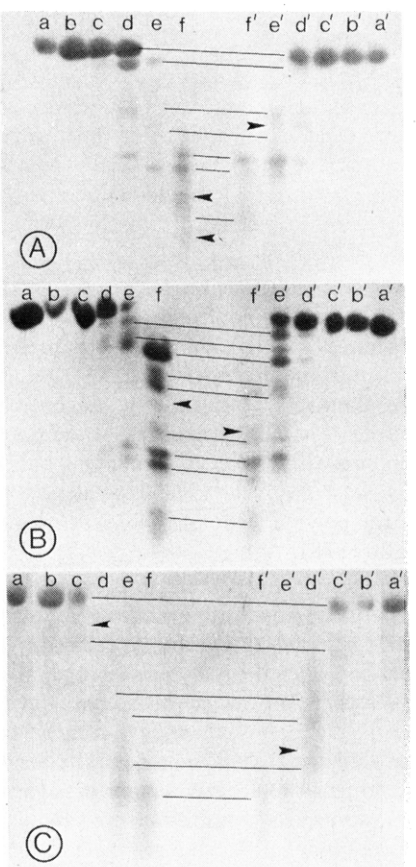

FIGURE 8: Fluorographs of Cleveland peptide maps of precursor and filaggrin. Precursor (a–f) and filaggrin (a′–f′) [^{3}H]histidine-labeled in vivo were digested by increasing protease concentrations (a–f) as described under Experimental Procedures. Elastase (A), *S. aureus* V8 protease (B), and ficin (C). Solid lines indicate bands found in both digests. Arrowheads show peptides produced from either protein that have no counterpart in the other protein digest.

S. aureus V8 protease both gave relatively simple peptide maps, suggesting restricted sites of cleavage under these conditions. On the other hand, ficin gave a complex map with few discrete peptides, perhaps because the enzyme is specific

for arginine (an abundant residue in these proteins) and is used close to its pH optimum. With each protease used, the peptide maps of filaggrin and the monomeric precursor show many similarities and several differences (arrows). Such patterns might be expected of two similar proteins, one of which has an activation peptide. It would also be expected if one of the proteins contained a prosthetic group, such as phosphate, that protected certain areas from cleavage.

Colorimetric analysis for hexose and hexosamine gave positive reactions for the precursor (approximately 2 mol/mol) and negative reactions for filaggrin. We sought to confirm these results by gas chromatography/mass spectral analysis but were unable to do so. The spectra were identical for the precursor and filaggrin and contained no recognizable sugars such as mannose, galactose, or their amino derivatives. Furthermore, we were unable to identify hexosamine in the precursor by ion-exchange chromatography on an amino acid analysis column. Thus, the colorimetric analysis for carbohydrate must be anomalous and due to some unknown moiety.

It is clear that the precursor protein is extracted as an oligomeric series whose molecular weights are multiples of the monomer (Figure 5B). These oligomers are very stable. The treatment of a precursor preparation containing both monomeric and polymeric species with either saturated EDTA or 2% β-mercaptoethanol for 18 h failed to elicit any change in the amount of the polymeric or monomeric species when analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (data not shown). The precursor proteins are also stable in 9% formic acid (Dale et al., 1980), and the monomeric protein that had been stored for 1 year at −20 °C in $NaDodSO_4$–polyacrylamide gel, after Cleveland peptide-mapping studies, showed no evidence of polymer formation when subjected to a second electrophoresis.

The individual precursor proteins are very stable and may be covalently linked but not by sulfhydryl groups. To test if the polymers were cross-linked by ester groups, we treated the polymeric form of the precursor with NH_4OH essentially as described by Lam & Kasper (1980). A generalized loss of protein (presumably by diffusion out of the polyacrylamide gel) was observed with prolonged incubation, but no formation of monomer was observed. The high degree of chemical stability of the polymers is consistent with the observations of Ball et al. (1978).

Attempted Dephosphorylation of the Precursor. The conversion of the precursor to filaggrin in vivo must take place at the same time that the granular cells are converted to cornified cells and when the lysosomes disrupt and release their enzymes. We therefore tested lysozomal acid phosphatase from epidermis for potential precursor-converting activity. This enzyme eluted at the void volume of the Sephacryl S-200 column and so has a molecular weight of $\geq$100 000. It has characteristics typical of a lysozomal enzyme, namely, it has a broad pH optimum between 4.5 and 5.6 in 0.1 M potassium phosphate–0.3% Triton X-100, it has a K_m of 0.11 ± 0.01 mM toward *p*-nitrophenol phosphate at pH 5.2, and it is inhibited completely by 0.5 mM sodium fluoride. It partly dephosphorylated the precursor, but even after prolonged incubation it failed to do so completely (Table II). Pretreatment of the precursor with trypsin allowed the release of slightly more phosphate by the phosphatase, but even so about 40% remained associated with the peptides or the protein. With pretreatment with trypsin, proportionately more phosphate was released by the phosphatase from the low M_r peptides than from those that elute in the void volume. Thus, the precursor may have a trypsin/phosphatase-resistant core. Epidermal phosphatase and commercially available acid and alkaline phosphatase were ineffective in converting the precursor to filaggrin when assayed by $NaDodSO_4$–polyacrylamide gel electrophoresis.

Table II: Acid Phosphatase Release of [^{32}P]Phosphate from Labeled Precursor[a]

treatment	% distribution of [^{32}P]phosphate: protein (V_0)	low M_r peptide (V_{sep})	inorganic phosphate (V_e)
no enzyme control	82	18	0
trypsin only control	48	39	13
phosphatase only	39	5	56
trypsin + phosphatase	26	13	61

[a] [^{32}P]phosphate-labeled precursor (250 μg) was incubated (20 h) with approximately 300 units of acid phosphatase from newborn rat epidermis and chromatographed on a Bio-Gel P-2 column as described in the text. The percentages of the specific activities in the peaks corresponding to the void volume, V_0 (i.e., proteins and peptides, M_r >2000), to the inorganic phosphate elution volume, V_e, and to the labeled peptides intermediate between these two extremes ($V_{sep} = V_e - V_0$) were determined from values obtained by scintillation counting. Separate samples were pretreated with trypsin as described in the text. Diisopropyl phosphorofluoridate-treated chymotrypsin and inorganic phosphate were used to determine V_0 and V_e, respectively.

Discussion

Filaggrin is a highly basic protein from the stratum corneum that causes aggregation of purified keratin filaments. The structure of the resultant macrofibrils resembles the keratin pattern in vivo (Dale et al., 1978). We have isolated a phosphorylated precursor of filaggrin and it fails to cause the aggregation of keratin filaments into these macrofibrils. This precursor thus has altered functional properties; it also differs in its size, its charge, and its tendency to aggregate. One or more of these altered characteristics may be a result of the presence of the large amount of phosphate (20 mol/mol) in this precursor. The monomeric form of the phosphorylated precursor, when analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis, has a relative molecular weight 5000 larger than that of filaggrin. It seems unlikely to us that this difference in molecular weight can be accounted for even by the maximum amount of phosphate assayed to be present in the molecule. It is possible that the phosphorylation causes the precursor to have an anomalously high apparent molecular weight when assayed by $NaDodSO_4$–polyacrylamide gel electrophoresis (Theurkauf & Vallee, 1982), but this does not appear to be the case in this particular system, because the phosphorylation of filaggrin by a cyclic nucleotide independent protein kinase does not result in an anomalous increase in molecular weight (M. Mamrack, personal communication). Furthermore, sedimentation equilibrium analysis, a more reliable method based on totally different principles from $NaDodSO_4$–polyacrylamide gel electrophoresis, also shows a difference of 5000 between the two proteins. Because the phosphate of the precursor should account for a maximum of only an M_r 2000 increase compared to that of filaggrin, the remaining molecular weight difference probably resides in the presence on the precursor of some as yet unidentified moiety. The similar but not identical amino acid compositions and peptide maps of the precursor and filaggrin are consistent with the presence of an extension peptide on the precursor. Conclusive evidence for the latter has proved elusive, since both

proteins have blocked N terminals and the same C-terminal residue (arginine).

This phosphorylated form of filaggrin is relatively insoluble, and evidence suggests that it accumulates in the amembranous keratohyalin granules of the granular cell layer (Dale & Ling, 1979b; Murozuka et al., 1979). These cells are rich in lysosomes [for a review, see Lazarus et al. (1975)]. Because the lysosomes rupture at the time of conversion of granular to cornified cells (the same time as the disappearance of the keratohyalin granules and the formation of filaggrin), lysosomal acid phosphatase might be expected to be involved in the generation of filaggrin. However, epidermal acid phosphatase only inefficiently removed phosphate from the purified precursor, and neither epidermal nor bacterial phosphatases were able to generate filaggrin from the precursor in vitro. Possibly the phosphate is buried within an enzyme-resistant core, as discussed below. Alternatively, if the precursor does contain an additional unidentified moiety as discussed earlier, it may be necessary to remove this moiety before conversion can occur. Thus, a number of enzymes and control mechanisms may be involved in the conversion process.

The complexity of the synthesis of filaggrin is further indicated by the observation that preparations of the precursor exist as a polymeric series. The precursor could be synthesized in the monomeric form (M_r (sed equil) = 44 200), which is later polymerized and covalently crosslinked, perhaps via ϵ-(γ-glutamyl)lysine bonds as described for cornified envelope proteins (Rice & Green, 1977). Transglutaminases, the enzymes involved in such cross-linking processes, have been identified in epidermal cells (Ogawa & Goldsmith, 1976; Peterson & Buxman, 1981). Alternatively, the precursor could be synthesized as a high molecular weight protein that is later cleaved into the monomeric and oligomeric forms. To resolve this problem we are currently identifying the product of translation of filaggrin mRNA in vitro.

The complexity of the synthesis of filaggrin is also demonstrated by a line of mutant mice that have a disorganized pattern of epidermal differentiation (Holbrook et al., 1982). These mutants show altered synthesis and phosphorylation of some of the keratins, and they also fail to convert a high molecular weight filaggrin cross-reactive protein to the M_r 27 000 mouse filaggrin. Thus, the whole process of keratinization is a highly organized process in which phosphorylation/dephosphorylation plays an important role.

In the case of the precursor of newborn rat filaggrin, we have shown that the phosphate is unevenly distributed throughout the molecule and may be localized to 30% or less of the molecule. In this situation, as many as one in six of the amino acids of this fragment contain phosphate. These phosphate moieties may be accomodated in clusters containing consecutive phosphoseryl residues, cf., phosvitin (Weller, 1974). However, unlike phosvitin, the precursor is not readily dephosphorylated by phosphatases with a broad specificity, and so the phosphate in the filaggrin precursor is probably buried and inaccessible to these enzymes. The localized distribution of phosphate in a specific region of the precursor may act to mask the filament binding region of filaggrin and so prevent premature aggregation of keratin filaments during epidermal differentiation.

Acknowledgments

We are grateful to Dr. W. Baschong and L. H. Ericsson for providing us, before publication, details of their method for the ethylamidation of proteins, to Dr. K. A. Holbrook for the electron microscopy, to S. B. Levery for GC/MS carbohydrate analysis, to R. Granberg for performing the amino acid analyses, and to W. Canfield for help with the preparative $NaDodSO_4$–polyacrylamide gel electrophoresis. Appreciation is also extended to J. A. Haugen Scofield and A. M. Lynley for their excellent technical help.

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Purification and Characterization of Troponin C from Pike Muscle: A Comparative Spectroscopic Study with Rabbit Skeletal Muscle Troponin C†

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ABSTRACT: The conformation of troponin C (TN-C) isolated from the white muscle of pike (*Esox lucius*), in the Ca^{2+} and metal-free states, was studied by circular dichroism, absorption difference spectroscopy, solvent perturbation difference spectroscopy, intrinsic fluorescence, thiol titration, and ^1H nuclear magnetic resonance spectroscopy. In addition, the molecular weight of the protein was determined by sedimentation equilibrium and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The composition of the protein was established by amino acid analysis. The resulting data were compared with those from the widely studied analogue isolated from rabbit skeletal muscle. The results indicate near equivalence in many of the properties of pike and rabbit TN-C, such as molecular weight, the magnitude of the calcium-induced conformational change, and urea- or thermal-induced denaturability. However, the pike protein has five additional potential carboxyl groups, and there is good evidence from NMR, solvent perturbation, and fluorescence studies for the presence of a buried tyrosine residue in the apo state.

Troponin C (TN-C),[1] the Ca^{2+}-binding subunit of the troponin complex from mammalian skeletal muscle, is an acidic protein, isoelectric point of 4.1–4.3, with a molecular weight as established from amino acid sequence studies of 17 965 (Collins et al., 1977). The molecule has no tryptophan residues but possesses a high ratio of phenylalanine to tyrosine residues, which results in a distinctive UV absorption spectrum similar to that noted for other Ca^{2+}-binding proteins such as parvalbumin and calmodulin.

An equilibrium dialysis study (Potter & Gergely, 1975) showed that TN-C has two distinct classes of Ca^{2+}-binding sites: two sites that have a high affinity for Ca^{2+} but also bind Mg^{2+} on a competitive basis (Ca^{2+}–Mg^{2+} sites, $K_{Ca^{2+}} = 2.1 \times 10^7\ M^{-1}$; $K_{Mg^{2+}} = 5 \times 10^3\ M^{-1}$) and two sites that bind Ca^{2+} specifically but with a lower affinity, $K_{Ca^{2+}} = 3.2 \times 10^5\ M^{-1}$. The four Ca^{2+}-binding sites have been numbered I–IV from the N terminus. A chemical modification study, which included the generation of peptide fragments (Sin et al., 1978), established that the two sites found closest to the N terminus are the low-affinity sites (I and II) and the high-affinity sites are located in the C-terminal half of the molecule (III and IV). This was further confirmed by Ca^{2+}-binding studies on specific fragments of the molecule (Leavis et al., 1978).

Ca^{2+} binding to TN-C has been studied by a number of spectroscopic techniques. These include an increase in the helical content of the protein, measured by CD (Murray & Kay, 1972), and changes in intrinsic tyrosine fluorescence (Kawasaki & van Eerd, 1972) and in changes in the environment of hydrophobic residues as probed by ^1H NMR (Levine et al., 1977; Seamon et al., 1977). It has generally been demonstrated that most of the structural changes take place when Ca^{2+} or Mg^{2+} bind to the high-affinity sites. Fluorescence studies employing the dansylaziridine probe at

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[1] Abbreviations: acto-HMM, a complex of fibrous actin and heavy meromyosin; ATPase, adenosinetriphosphatase; CD, circular dichroism; DEAE, diethylaminoethyl; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; pCa^{2+}, the negative logarithm of the free calcium ion concentration; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Mops, 4-morpholinepropanesulfonic acid; Pipes, piperazine-*N,N′*-bis(2-ethanesulfonic acid); UV, ultraviolet; TN-C, troponin C; TN-I, troponin I; r^2, the square of the radial distance; Me_2SO, dimethyl sulfoxide; NMR, nuclear magnetic resonance; DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; M_r, molecular weight.

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