Isolation and Characterization of Titin T1 from Bovine Cardiac Muscle†

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ABSTRACT: Methods were developed for isolation of bovine cardiac titin in the apparently full-sized T1 form. The pure protein could be obtained either in the denatured state after chromatography of myofibril extracts on Sephacryl S-1000 or in the native state using hydroxylapatite chromatography. Titin-specific chicken polyclonal antibodies against denatured titin stained the I band regions and weakly stained the M line area of cardiac myofibrils but primarily stained the A–I junction positions in skeletal muscle myofibrils. Bovine cardiac titin contained 176 ± 5 μmol of sulfhydryl groups per gram in the presence of 6 M guanidine hydrochloride or 0.1% SDS; only 45% of these groups were reactive under non-denaturing condition. Circular dichroism measurements indicated that titin contained approximately 53% β-sheet and 47% random coil at pH 7.0. These results are consistent with the idea that titin is primarily composed of immunoglobulin C2 and fibronectin type III like motifs throughout its length, including the flexible I band region.

Titin (also called connectin) is an extremely large myofibrillar protein with a subunit weight of 2 to >3 × 10^6 D (Maruyama et al., 1984; Kurzban & Wang, 1988; Higuchi et al., 1993) which is believed to span from the M line to the Z line in the sarcomere (Maruyama et al., 1985; Furst et al., 1988; Vinkemeier et al., 1993). It is proposed to play a role in maintaining the A band in the center of the sarcomere during force development (Horowits & Podolsky, 1987, 1988) and may function to align the thick and thin filaments during myofibrillogenesis (Wang et al., 1988; Furst et al., 1989; Schultheiss et al., 1990; Handel et al., 1991). A part of the cDNA sequence for titin has been obtained (Labeit et al., 1990, 1992; Maruyama et al., 1993, 1994; Tan et al., 1993; Gautel et al., 1993a,b), and it mostly encodes a series of approximately 100 amino acid repeating motifs of two types. The cDNA sequence for rabbit cardiac titin has been found to be essentially identical to that for skeletal muscle in the regions examined to date (Fritz et al., 1991, 1993).

Titin from skeletal muscle usually appears as a doublet on SDS[1] gels, and the lower band (T2 or β-connectin) is thought to represent a proteolytic breakdown product of the upper band (T1 or α-connectin) (Wang, 1982). Several groups have purified titin in its native state from high ionic strength extracts of skeletal and cardiac muscle (Maruyama et al., 1984; Trinick et al., 1984; Wang et al., 1984; Gassner, 1986; Itoh et al., 1986; Nave et al., 1989). However, the primary component in most of these preparations is the smaller T2 species. Recently a method has been described to purify the larger T1 component of rabbit skeletal muscle, but the procedures employ chromatography in 4 M urea, yield only a small proportion of the total titin as T1, and result in a preparation that rapidly degrades during storage (Kimura & Maruyama, 1989; Kimura et al., 1992).

Since most of the previous titin purification schemes have yielded the shorter T2 species, we attempted to purify titin as a full-length protein. Methods for purification of titin from cardiac muscle consisting predominantly of T1 are described, and some of the properties of this protein are reported and compared to those reported previously for the truncated titin T2.

MATERIALS AND METHODS

Purification of SDS–Urea Solubilized Titin. Cardiac myofibrils were used as a starting material for the purification of denatured titin. To prepare purified myofibrils, all steps were performed at 0–4 °C except where noted. Bovine hearts, obtained approximately 30 min after death, were immersed in ice, and the left ventricle was removed and trimmed of excess fat and connective tissue. The cardiac muscle (250 g) was homogenized in a Waring blender for 1 min in 2.5 L of rigor-Triton buffer containing 75 mM KCl/2 mM EGTA/2 mM $MgCl_2$/0.5% Triton X-100/15 mM β-mercaptoethanol/0.1 mM phenylmethanesulfonyl fluoride (PMSF)/2 mM NaN_3/6 mM phosphate (pH 7.2). The homogenate was stirred for 10 min and centrifuged at 3500*g* for 5 min. The precipitates were resuspended, homogenized, and centrifuged several times (usually three times) until the red color was gone. The residue was then resuspended and filtered through one layer of cheese cloth to remove connective tissue pieces, and the mixture was centrifuged at 3500*g* for 5 min. The whitish cake of myofibrils was washed in rigor-Triton as above one more time. The preparation was then washed two times with Triton X-100-free rigor buffer and resuspended in 75 mL of rigor-DTT (1 mM) buffer. After mixing with 2 vol of 75% glycerol in rigor-DTT buffer, the myofibril suspension was stored at −20 °C until use.

To purify titin, an aliquot of the glycerinated myofibrillar suspension (approximately 0.6 g of protein) was washed two times with 6 mM phosphate (pH 7.5)/5 mM EDTA and

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[1] Abbreviations: SDS, sodium dodecyl sulfate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; DTT, dithiothreitol; Temed, *N,N,N′,N′*-tetramethylethylenediamine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, (ethylenedinitrilo)tetraacetic acid.

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centrifuged at 3500*g* for 5 min. The final pellet was dissolved in 50 mL of a solution containing 6.4 M urea/1 M thiourea/5 mM EDTA/2.5% SDS/50 mM DTT/50 mM sodium phosphate (pH 7.5). The suspension was heated in boiling water for 5 min and centrifuged at 15000*g* for 30 min at room temperature (almost all myofibrillar proteins were solubilized by this treatment; only a small pellet was obtained after centrifugation). The clear supernatant was applied to a Sephacryl S-1000 column (5 × 85 cm) which had been equilibrated at room temperature with 6.4 M urea/0.2% SDS/1 mM DTT/2.5 mM EDTA/50 mM sodium phosphate (pH 7.5). Titin eluted from the column as the first protein peak without need for further purification (the first 280-nm-absorbing peak had a high A_{260}/A_{280} ratio, typical for nucleic acids). It was found that most of the titin becomes insoluble during overnight storage at 4 °C. The suspension was then centrifuged at 15000*g* for 20 min at −10 °C. The collected pellet was liquefied by warming to room temperature and found to contain 75–80% of the titin from the original suspension. In this way titin was concentrated about 10 times (from 0.15 to 1.5 mg/mL).

At this stage of purification, 25–30 mg of titin was obtained from 0.6 g of cardiac myofibrils. Before further studies, excess SDS was removed from the titin solution by dialyzing against 6 mM sodium phosphate (pH 7.2)/1 mM DDT/1 mM NaN_3/0.005% SDS at room temperature.

Purification of Native Titin. The procedure for the preparation of native cardiac titin was modified from that of Kimura and Maruyama (1983) who used chicken breast muscle. Bovine ventricle tissue (300 g, prepared as above before the homogenization step) was homogenized in 10 vol (v/w) of 25 mM phosphate (pH 7.2)/1 mM NaN_3 (4 °C) with a blender (containing a Polytron type rotor–stator in an inverted Ball jar on a Waring blender base) for 30 s with a 15-s break every 10 s. The suspension was stirred with an overhead paddle for 15 min and then centrifuged at 3500*g* for 5 min, and the supernatant was discarded. Pellets were resuspended and homogenized for 10 s in fresh solution. This washing procedure was repeated two more times. After the last homogenization, the myofibrillar suspension was filtered through one layer of cheesecloth (to remove connective tissue) before centrifugation. The final pellet was resuspended in 10 vol of 5 mM $NaHCO_3$/1 mM NaN_3/15 mM β-mercaptoethanol, stirred for 15–45 min, and then centrifuged at 10000*g* for 10 min. This was repeated three more times. At the fourth resuspension, 0.5% Triton X-100 was also included in the buffer. To the washed residues was added 10 vol of 0.1 M potassium phosphate (pH 5.8)/1 mM NaN_3, and the mixture was stirred for 45 min. The myofibrils were sedimented (10000*g* for 10 min) and extracted one more time with the same solution. After washing with 50 mM potassium phosphate (pH 6.6)/1 mM NaN_3, the final residue was extracted with 2 vol of 0.15 M potassium phosphate (pH 6.6)/1 mM DDT/1 mM NaN_3 for 10 min at 4 °C. The suspension was centrifuged at 15000*g* for 20 min, and the supernatant was filtered through glass wool. The filtrate was applied to a hydroxylapatite column (4 × 7 cm) and the proteins subsequently eluted (details described in the legend to Figure 1). Pooled titin fractions were mixed with saturated ammonium sulfate to bring the final solution to 25% saturation, and the mixture was centrifuged (the pellets contained contaminating proteins and/or titin fragments). The supernatant solution was then brought to 40% saturation to precipitate the titin. The final pellet was resuspended in 0.5 M potassium phosphate (pH 7.0)/30 mM β-mercaptoethanol, dialyzed versus 0.25 M potassium phosphate (pH 7.0)/30 mM β-mercaptoethanol overnight, and subsequently dialyzed versus 0.1 M sodium phosphate (pH 7.0)/1 mM DDT/1 mM NaN_3 or another high ionic strength solution. Direct dialysis of ammonium sulfate-precipitated titin against 0.1 M sodium phosphate resulted in abnormally high A_{280} readings due to protein turbidity (measured by light scattering at 320 nm). The gradual ionic strength changes as described above thus markedly reduced the problems with protein aggregation.

All steps through the application of the crude titin extract on the hydroxylapatite column were performed in 1 day. Some titin preparations contained myosin after the hydroxylapatite column. With these the mixture was ammonium sulfate fractionated and dialyzed as described above except that the final dialysis was versus 0.10 M potassium phosphate (pH 7.5)/1 mM DTT/1 mM NaN_3. The titin–myosin mixture was applied to a 10-mL DEAE-Sephadex A-50 column equilibrated with the same solution. The titin emerged from this column in the void volume, while the myosin was retained.

SDS–Polyacrylamide Gel Electrophoresis. Protein samples were analyzed by discontinuous SDS–gel electrophoresis in a Hoefer SE250 Mighty Small Unit according to Laemmli (1970) as modified by Fritz et al. (1989). These modifications include the use of a thiourea–urea–SDS containing sample buffer for reproducible titin solubilization, a 3% stacking gel using *N,N'*-diallyltartardiamide as the cross-linker, a 10% polyacrylamide separating gel (200:1 ratio of acrylamide to *N,N'*-methylenebis(acrylamide)), and a separating gel buffer at pH 9.3 with double the concentration of Tris versus the typical Laemmli recipe. After electrophoresis, gels were stained in Coomassie Brilliant Blue R-250. For easier handling of gels and enhanced sensitivity in visualizing the protein bands, fully destained gels were size-reduced in 13% (w/w) poly(ethylene glycol) 8000 (Palumbo & Tecce, 1983).

Amino Acid Analysis. Protein samples (about 50 μg) were hydrolyzed in 6 N HCl with or without 0.4% (v/v) β-mercaptoethanol for 22 h at 110 °C in sealed evacuated tubes (Ng et al., 1987). The PTC-amino acids were prepared as previously described (Bidlingmeyer et al., 1984). Amino acid analysis was performed by high-performance liquid chromatography using a Pico-Tag column (Waters). Cysteic acid was determined after hydrolysis of the protein in 6 N HCl containing 0.28 M dimethyl sulfoxide (Spencer & Wold, 1969), and tryptophan was determined after hydrolysis in 4 M methanesulfonic acid containing 0.1% (w/v) tryptamine (Cohen et al., 1987).

Protein Determination. Bovine cardiac titin concentration was measured by absorbance at 280 nm (after correction for light scattering at 320 nm) using a specific absorption coefficient of 1.51 L g^{-1} cm^{-1} (protein concentration for calibration was determined by amino acid analysis). The protein concentration of myofibrils was determined by the bicinchoninic acid assay of Smith et al. (1985) using bovine serum albumin (BSA) as a standard.

DTNB Assay. Ellman's reagent (DTNB) was used to determine free thiol groups in denatured and native titin using absorbance readings at 412 nm (Ellman, 1959). Total cysteine content of purified titin was measured in 6 M guanidine hydrochloride/50 mM Tris (pH 8.0)/1 mM EDTA for denaturation. To detect the effect of SDS on the accessibility of DTNB-reactive groups, DTNB was first reacted with purified, native titin (about 0.25 mg/mL) in 0.1 M sodium phosphate (pH 7.0)/1 mM EDTA for 20 min after the A_{412} readings had reached a plateau. Subsequently SDS was added to the reaction mixture to make a final concentration of 0.1%, and the reaction was recorded for another 20 min. In calculating the concentration of sulfhydryl groups, a molar

absorption coefficient of 13 600 M^{-1} cm^{-1} at 412 nm was used for determinations in 6 M guanidine hydrochloride, while 14 150 M^{-1} cm^{-1} was employed for determinations in 0.1 M phosphate solutions (Riddles et al., 1979).

Indirect Immunofluorescence. Bovine rectus abdominus and cardiac muscle was obtained 30 min postmortem and myofibrils isolated as previously described (Pan et al., 1986). Myofibrils were gently smeared over coverslips and processed for immunofluorescence staining (Handel et al., 1991) using a primary antibody concentration of 15–30 μg/mL. After secondary antibody incubation and a final wash with rigor buffer, the myofibrils were mounted in 70% glycerol/rigor buffer containing *p*-phenylenediamine (1 mg/mL). Control samples without primary antibody were run in parallel. The slides were viewed with a Nikon Diaphot microscope equipped for phase contrast and epifluorescence illumination. Images were obtained using a 100× objective (NA 1.4) and a CCD camera (Thomson 7883) controlled via a Macintosh IIfx with a Matrox board using Nu200 2.0 software (Photometrics). Exposure times were typically 0.5–1 s. Images were processed using Image 1.38 software (Wayne Rasband, NIH), photographed from the computer monitor with a 35 mm camera and 2415 film, and developed with HC110 dilution F.

Chicken Immunization and Isolation of Antibody. Purified denatured titin was dialyzed against 6 mM sodium phosphate (pH 7.2)/1 mM DDT/0.005% SDS to remove excess SDS before immunization. Native titin was in 0.1 M sodium phosphate (pH 7.2). Hens were immunized intramuscularly with 0.25 mg of titin in Freund's complete adjuvant at multiple sites; this was followed by another injection 10 days later. Eggs were collected before immunization (for preimmune antibodies) and beginning 10 days after the second injection. Antibodies were purified from the egg yolk as previously described (Polson et al., 1980).

Immunoblotting Assay. Protein samples were subjected to SDS–polyacrylamide gel electrophoresis as described above, and the resolved proteins were then electrophoretically transferred onto nitrocellulose (Schleicher & Schuell) or Immobilon-P membranes (Millipore). The procedure for transfer is similar to that of Towbin et al. (1979) except that methanol was reduced to 15% and 10 mM β-mercaptoethanol and 0.005% (w/v) SDS were included in the transfer buffer (Fritz et al., 1989). Two gels having identical protein samples were transferred at the same time using a constant current of 0.4 A for 2 h. One membrane was stained with 0.1% India ink for visualizing protein bands (Hancock & Tsang, 1983). The other one was sequentially blocked in BSA, incubated with primary antibody, washed, treated with peroxidase-labeled secondary antibody (Bio-Rad; 1:3000 dilution), washed, and incubated with 4-chloro-1-naphthol substrate. Blots were photographed using Kodak 4415 film, which was developed in Dektol for 3 min.

Sample Preparation for Circular Dichroism Measurements. Purified native titin (0.5–0.7 mg/mL) was dialyzed against various salt solutions overnight. Titin concentration was measured by absorbance at 280 nm after correction for light scattering at 320 nm as described above. In all cases, 1 mM DDT was included in the dialysis solutions. Protein samples were then diluted to a concentration of 0.1–0.25 mg/mL and centrifuged at 15000*g* for 15 min. Each sample was degassed for 10 min with an aspirator immediately prior to circular dichroism (CD) measurements. Scans were made in a modified and computerized CARY Model 60 spectropolarimeter (On-line Instrument Systems, Inc., Jefferson, GA). The instrument was calibrated using *d*(+)-10-camphorsulfonic acid, and a 0.1-cm cell was used. Ten scans of each sample were averaged, and the mean residue ellipticity [Θ] was expressed as deg cm^2 $dmol^{-1}$. The mean residue molecular weight of bovine cardiac titin was 112 as calculated from the amino acid composition. All spectra were corrected for the proper baseline using the final dialysis solution in each case. Secondary structure proportions (α-helix, β-sheet, β-turn, and random coil) were analyzed by the method of Chang et al. (1978), which uses reference spectra from 15 proteins with known secondary structures. The simulation was done in the wavelength region of 190–240 nm at 1-nm intervals.

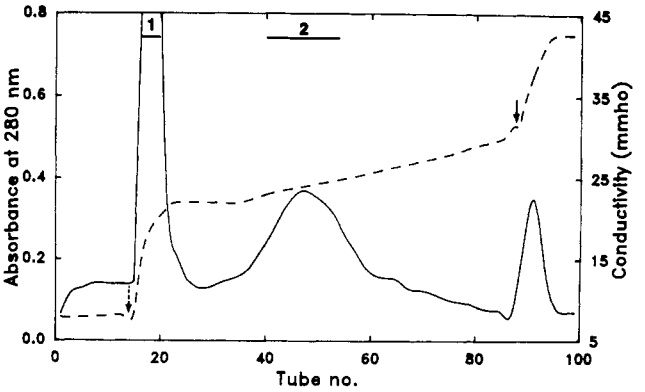

FIGURE 1: Hydroxylapatite chromatography of native cardiac titin. The 0.15 M potassium phosphate (pH 6.6)/1 mM DDT extract was centrifuged at 15000*g* for 20 min, filtered through glass wool, and applied to a hydroxylapatite column (4 × 7 cm) equilibrated with the same buffer. The column was first washed with 150 mL of 0.2 M KCl/0.15 M potassium phosphate (pH 7.0)/1 mM DDT/1 mM NaN_3 (dashed arrow) and then eluted with a continuous gradient of 250 mL each of 0.2 M KCl/0.15 M potassium phosphate (pH 7.0)/1 mM DDT/1 mM NaN_3 and 0.2 M KCl/0.3 M potassium phosphate (pH 7.0)/1 mM DDT/1 mM NaN_3. After the gradient was finished, the column was washed with 150 mL of 0.2 M KCl/0.5 M potassium phosphate (pH 7.0)/1 mM DDT/1 mM NaN_3 to remove all material remaining on the column (solid arrow). The flow rate was 120 mL/h, and 8-mL fractions were collected. The bars indicate the tubes pooled for each fraction: fraction 1, tubes 17–20; fraction 2, tubes 40–54.

RESULTS

Purification of Native Titin. The chromatographic profile of extracts of native titin on hydroxylapatite is shown in Figure 1. The first peak was eluted with 0.2 M KCl/0.15 M potassium phosphate (pH 7.0) and contained C-protein and other low molecular weight proteins (Figure 2e). The titin was released after the phosphate gradient started, and the peak appeared at 0.2 M KCl/0.18 M potassium phosphate (pH 7.0). There was slight contamination with other proteins and/or titin degradation products (Figure 2f); these contaminants were separated from titin by precipitation with 25% ammonium sulfate saturation (Figure 2g). Approximately 30 mg of native titin was obtained from 300 g of cardiac muscle by this procedure.

Purified Bovine Cardiac Titin Is in T1 Form. When bovine cardiac muscle tissue or purified myofibrils were analyzed by SDS–gel electrophoresis, only one titin band was found (Figure 3c,d). However, bovine skeletal myofibrils, prepared by the same method as used to purify cardiac myofibrils, had two high molecular weight bands (Figure 3a) corresponding to T1 and T2 in rabbit skeletal myofibrils (Wang, 1982). The upper band (T1) appeared to comigrate with the only titin band in cardiac muscle and purified myofibrils. Furthermore, fresh skeletal muscle tissue electrophoresed on the gel showed one titin band (T1) instead of a doublet band in the skeletal myofibrils (Figure 3b). Thus the observed differences suggest that cardiac titin is less subject to proteolysis than skeletal muscle titin during the purification procedure. Bovine cardiac denatured or native titin purified by our methods appeared

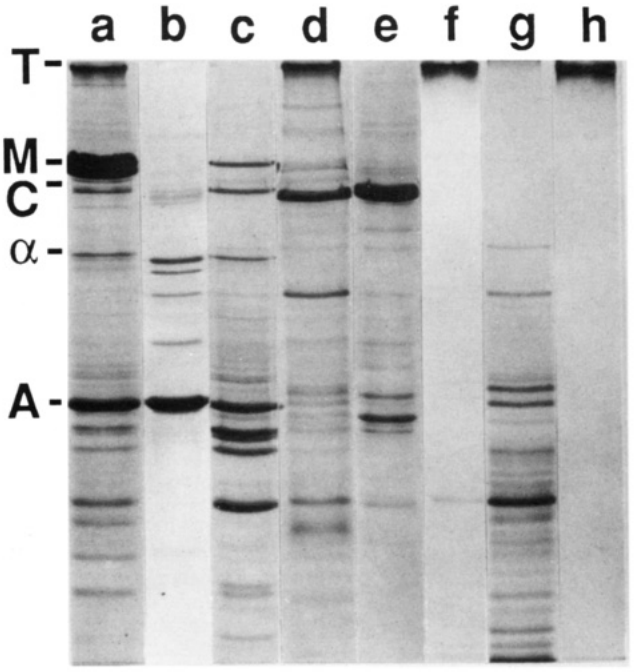

FIGURE 2: SDS–gel electrophoresis patterns of fractions during native titin purification: (a) purified bovine cardiac myofibrils, 11 μg; (b) 5 mM $NaHCO_3$/1 mM NaN_3/15 mM β-mercaptoethanol soluble fraction, 4 μg; (c) 0.1 M potassium phosphate (pH 5.8) soluble fraction, 6 μg; (d) 0.15 M phosphate (pH 6.6) soluble fraction applied to the hydroxylapatite column, 4 μg; (e) fraction 1 from hydroxylapatite (Figure 1), 3 μg; (f) fraction 2 from hydroxylapatite (Figure 1), 2 μg; (g) precipitate from fraction 2 with 25% ammonium sulfate saturation, 4 μg; (h) purified titin, 3 μg.

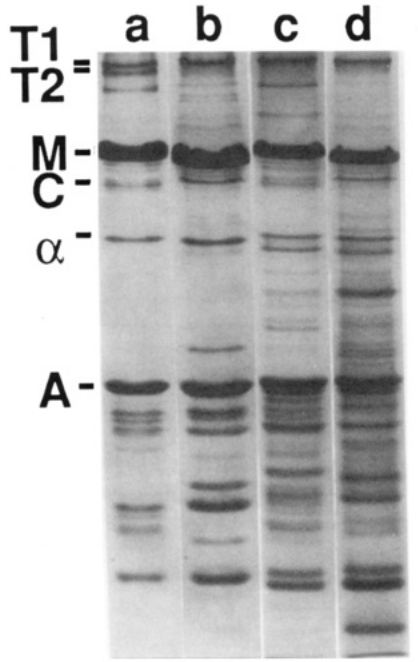

FIGURE 3: SDS–gel electrophoresis patterns of bovine whole muscle tissues and purified myofibrils. Bovine skeletal whole muscle (rectus abdominus) and cardiac whole muscle taken immediately after death were frozen in liquid nitrogen and pulverized with a mortar and pestle. Cardiac and skeletal muscle myofibrils were prepared as described in Materials and Methods using the steps before SDS–urea solubilization. Samples were solubilized in sample buffer and applied to SDS–polyacrylamide gels. The sample sources and amounts applied to the gels were (a) skeletal muscle myofibrils, 8 μg; (b) cardiac myofibrils, 13 μg; (c) skeletal muscle, 11 μg; and (d) cardiac muscle, 13 μg.

as one band on SDS gels and comigrated with the only titin band from purified cardiac myofibrils (Figure 2). It is thus predominantly the full-sized T1 type.

Table 1: Amino Acid Compositions of Titin from Bovine Cardiac Muscle as Compared with Those from Other Tissues and Species

amino acid[a]	bovine cardiac native[b]	bovine cardiac SDS–urea[b]	bovine[c] skeletal	porcine[d] cardiac	chicken[e] breast	rabbit[f] skeletal
Asx	84	84	83	89	96	95
Glx	103	106	115	112	111	136
Ser	64	69	67	60	60	73
Gly	74	79	69	81	74	65
His	17	19	15	16	15	17
Arg	61	63	49	54	59	44
Thr	64	70	68	69	76	78
αla	69	69	65	77	65	66
Pro	62	65	82	66	74	74
Tyr	41	43	29	31	31	28
Val	73	80	92	86	87	74
Met	15	17	12	14	12	8
Cys/2	20[g]	18	13	3	2	8
Ile	52	54	55	57	60	44
Leu	75	74	61	74	66	65
Phe	35	32	26	29	26	24
Trp	20[h]	nd[i]	13	ND	ND	ND
Lys	71	58	86	82	86	101

[a] Number of residues per 1000 residues. [b] Average from four hydrolysates. [c] From Lusby et al. (1983). [d] From Itoh et al. (1986). [e] From Kimura and Maruyama (1983). [f] From Wang (1985). [g] Determined as cysteic acid after hydrolysis in 6 N HCl containing 0.28 M dimethyl sulfoxide (Spencer & Wold, 1969). [h] Determined after hydrolysis in 4 M methanesulfonic acid containing 0.1% (w/v) tryptamine (Cohen et al., 1987). [i] Not determined.

Amino Acid Composition. The amino acid compositions of native and denatured bovine cardiac titins were very similar (Table 1). The lower content of lysine in denatured titin was probably due to losses from reaction with cyanate (derived from urea in the buffer) during solubilization and gel filtration. On the whole, bovine cardiac titin is rich in nonpolar amino acids (about 40%) and has a rather high content of proline (about 6%). In addition, the percentages of tyrosine (4%) and tryptophan (2%) result in a specific absorption coefficient (1.51 L g^{-1} cm^{-1} at 280 nm) which is higher than that of most other myofibrillar proteins.

A comparison of the amino acid composition of native bovine cardiac titin with that of porcine cardiac titin shows that there are small differences in the contents of tyrosine, phenylalanine, valine, and lysine (Table 1). In contrast there was approximately 6-fold more cysteine in bovine cardiac titin versus the porcine cardiac counterpart. However, for the remainder of the amino acids the composition was more similar between bovine cardiac and porcine cardiac than with titins from bovine, chicken, or rabbit skeletal muscle (Table 1).

Sulfhydryl Content. The total sulfhydryl content of bovine cardiac titin was determined in the presence of 6 M guanidine hydrochloride by DTNB assay. Our results showed 176 ± 5 μmol of sulfhydryl groups/(gram of titin) (six determinations on three different preparations). If a mean residue weight of 112 is assumed (derived from the amino acid composition of titin), the above value corresponds to 19.7 ± 0.6 sulfhydryl groups per 1000 amino acid residues. This is consistent with the amino acid analysis value of 20 cysteines per 1000 amino acid residues (Table 1). When measured in the absence of SDS, native titin appeared to have 79 ± 3 μmol of sulfhydryl groups/(gram of titin) (four determinations on two different preparations) available to react with the DTNB reagent. Thus 55% of the total sulfhydryl content is buried in native titin.

Antibody Specificity. Chicken antibody raised against denatured bovine cardiac titin was characterized by immunoblotting. The specificity of the anti-titin is readily apparent (Figure 4). Only the titin band was stained on the immunoblot containing cardiac myofibrillar proteins (Figure 4c). This was confirmed by the fact that purified titin run in parallel

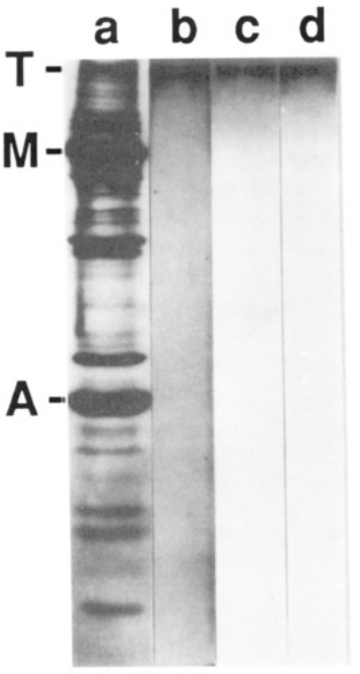

FIGURE 4: Characterization of anti-titin antibody. Protein samples were resolved on an SDS–10% polyacrylamide gel and electrophoretically transferred to an Immobilon-P membrane. One part of the membrane was stained with India ink (lanes a and b), while the other (lanes c and d) was immunologically probed with anti-titin (40 μg/mL) and peroxidase-conjugated secondary antibody (Bio-Rad; 1:3000 dilution) as described in Materials and Methods. Lanes a and c, bovine cardiac myofibrils; lanes b and d, purified native bovine cardiac titin.

also showed strong peroxidase staining (Figure 4d). The same specificity was obtained using antibody concentrations from 1 to 400 μg/mL; 40 μg/mL was used routinely (Figure 4). Similar results were obtained using antibody to native titin.

The cross-reactivity patterns of anti-titin with rabbit cardiac, rabbit skeletal, and bovine skeletal muscles were also tested by immunoblotting. All samples incubated with anti-titin showed only immunoreactivity with the titin band (data not shown).

Immunofluorescence Study. Bovine cardiac myofibrils were stained by indirect immunofluorescence using anti-denatured titin and rhodamine-labeled secondary antibody. Intense staining was associated with the I bands in the sarcomere (Figure 5a) with a weaker stained zone near the middle of the A band. Bovine skeletal muscle myofibrils were also stained with the antibody. Immunofluorescent labeling was most intense at the A–I junction, faint in the rest of the half–I band, and virtually absent at the Z line (Figure 5c). There appeared to be no additional staining intensity in the middle of the A band. Control slides of both cardiac and skeletal muscle myofibrils that were only incubated with secondary antibody had virtually no staining (intensities more than 20-fold less than those including primary antibodies).

Circular Dichroism Spectra of Native Protein. The far-ultraviolet CD spectrum (190–240 nm) of native bovine cardiac titin in 0.1 M potassium phosphate (pH 7.0)/1 mM DTT showed a negative trough at 201 nm (Figure 6), with a mean residue ellipticity of −16 000 deg cm^2 dmol^{-1}. The results showed that native titin consists of 53% β-sheet and 47% random coil. CD measurements on native titin which had been dialyzed versus 6 M guanidine HCl/0.1 M potassium phosphate (pH 8.5)/1 mM DTT yielded spectra consistent with 100% random coil.

The effects of ionic strength and pH on the secondary structure of titin were investigated. Higher concentrations of

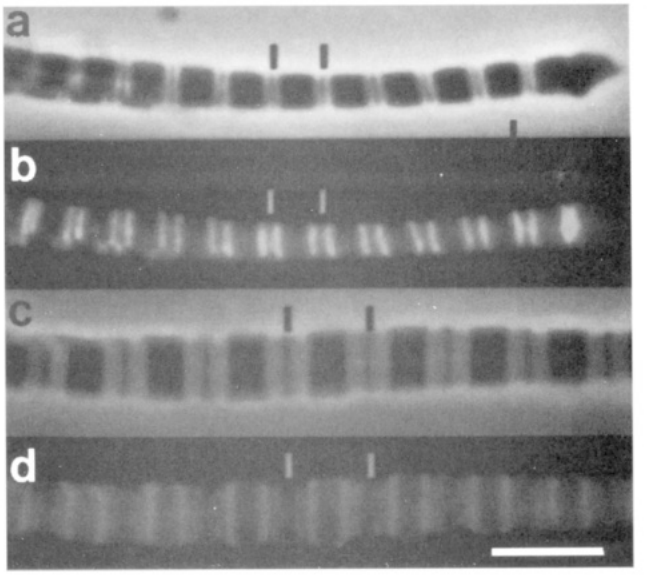

FIGURE 5: Indirect immunofluorescence of bovine cardiac and skeletal muscle myofibrils stained with anti-titin. a and c, phase contrast. b and d, fluorescence image. Lanes a and b, bovine cardiac myofibril. Intense staining was found in the I bands on either side of the Z line (vertical bars). Weaker staining was found near the middle of the A band. Lanes c and d, bovine skeletal muscle myofibril. The anti-titin labeled primarily the A–I junction regions of the myofibrils. Bar, 5 μm.

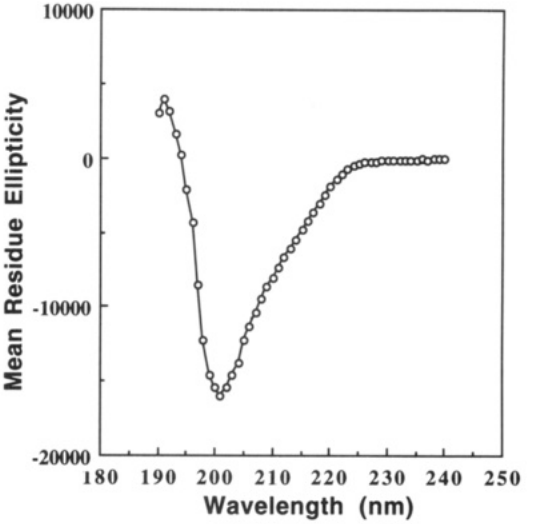

FIGURE 6: Circular dichroism spectrum of native bovine cardiac titin in 0.1 M potassium phosphate (pH 7.0)/1 mM DTT.

phosphate buffer (0.2 and 0.3 M) only slightly increased the negative ellipticity. Titin in 0.5 M KCl/50 mM phosphate (pH 7.0)/1 mM DDT gave a secondary structure distribution similar to those obtained in buffers containing only the phosphate anion. Thus variations in ionic strength resulted in negligible differences in the secondary structure of titin as determined by CD measurements. The CD spectra of titin in the pH range from 7.0 to 8.5 also gave similar β-sheet and random coil proportions with no indication of α-helix content.

DISCUSSION

Methods are described for the isolation of bovine cardiac titin as the apparently full-length T1 form in either the denatured or the so-called "native" state. T2, a presumed proteolytic product of T1, has been isolated from chicken breast, rabbit skeletal, and porcine cardiac muscles in a native form (Maruyama et al., 1984; Trinick et al., 1984; Wang et al., 1984; Itoh et al., 1986; Nave et al., 1989). Kimura and co-workers (1992) have described the purification of α-con-

nectin (the equivalent of titin T1) from rabbit skeletal muscle. However, the procedure employed 4 M urea during a chromatography step, the yields were low (4 mg/(100 g of muscle)), and the preparation was rapidly degraded during storage. Soteriou and co-workers (1993) have recently described a rapid procedure for purification of "relatively undegraded titin" which is primarily the larger titin species (T1). However, contaminating components of 160 and 100 kDa were consistently found in these preparations. Thus there are no previously published procedures for purification of a homogeneous, full-length titin without use of denaturing solvents. Since the missing segment may exceed 500 kDa in size, full characterization of the protein will require study of the nontruncated form.

Native titin contains 20 cysteines per 1000 residues as determined by amino acid analysis after protein hydrolysis in the presence of dimethyl sulfoxide (Table 1). This value is considerably higher than that found previously (3/1000) with porcine cardiac titin (Itoh et al., 1986). This difference may be due to the species or to the fact that the porcine cardiac protein was the truncated β-connectin (T1). DTNB assay in the presence of SDS gave a total cysteine content similar to that measured by amino acid analysis. However, only 45% of the total sulfhydryl content was found to react with DTNB in the absence of SDS (i.e., the native protein). Thus, in spite of titin's apparent flexibility, a considerable portion of the sulfhydryl groups remain inaccessible when the protein is in the native state. The internal location of several cysteines has also been noted in telokin, a small gizzard muscle protein with a structure similar to immunoglobulin C2 domains (Holden et al., 1992).

Monoclonal antibodies raised against bovine cardiac (Wang & Greaser, 1985), chicken cardiac (Hill & Weber, 1986), chicken skeletal (Furst et al., 1988, 1989), or rabbit skeletal titin (Whiting et al., 1989) cross-react with titins in a large number of species. Polyclonal antibodies against chicken skeletal muscle titin were found to cross-react with porcine cardiac titin and frog skeletal muscle titin (Maruyama et al., 1985; Itoh et al., 1986). It was therefore not surprising that chicken antibodies generated against bovine cardiac titin showed immunological cross-reactivities with cardiac or skeletal muscle titin from several species. Work on the cDNA sequence of rabbit skeletal and rabbit cardiac titin indicates regions of identity (Labeit et al., 1990, 1992; Fritz et al., 1991, 1993); this suggests that the titin isoforms arise from alternative splicing similar to that found in several other contractile proteins.

Immunofluorescence studies with our polyclonal anti-titin revealed that the half-I bands and the M line regions of cardiac myofibrils were the primary sites of labeling. Previous results obtained with titin monoclonal antibodies have demonstrated that titin extends from the M line to the Z line (Fürst et al., 1988, 1989). Thus one would expect that staining with a polyclonal antibody should cover the entire half-sarcomere. The restricted regions of labeling observed suggest that (1) different segments of titin are not uniformly antigenic or (2) parts of the epitopes are shielded in the myofibril structure. The fact that there are a large number of titin monoclonal binding sites scattered throughout the A band, the I band, and the Z lines argues against the second possibility.

The staining patterns of skeletal muscle myofibrils were different with primarily the A–I junction positions being labeled (see Figure 5). These differences in staining cannot be ascribed to the sarcomere length differences between cardiac and skeletal muscle myofibrils; the occasional longer cardiac myofibril observed had wide, intense I band staining, while shortened skeletal muscle myofibrils were weakly stained with most of the fluorescence localized at the A–I junctions (data not shown). The differences in staining positions, particularly in the I band region and the M line region, between cardiac and skeletal muscle myofibrils suggest that either there are tissue-specific isoforms of titin or there are structural differences in the myofibrils which result in variable exposure of the antigenic sites. Hill and Weber (1986) concluded that skeletal and cardiac titin were immunologically different using immunofluorescence and immunoblotting assays. Extensive polymorphism with resulting isoforms exists within the family of "muscle" proteins (Zak, 1981). Wang and co-workers (1991) and Horowits (1992) have shown that different-sized isoforms of titin exist in skeletal muscle. Our current immunofluorescence results provide further evidence for the existence of cardiac and skeletal muscle titin isoforms. However, although other studies suggest that rabbit cardiac titin is smaller than rabbit skeletal titin T1 (Wang et al., 1991, 1993), the current work shows no apparent size difference between bovine cardiac and skeletal muscle T1 titin (Figure 3b,d).

It was previously reported that titin which has been ammonium sulfate precipitated becomes permanently aggregated (Nave et al., 1989). In the present work it was found that dialyzing ammonium sulfate-precipitated titin directly against 0.5 M potassium chloride or ammonium acetate resulted in significant turbidity even with protein concentrations in the 1–2 mg/mL range. However, native titin could be prepared with minimal turbidity (measured by absorbance at 320 nm) if the solubilization and dialysis protocol outlined was used. The use of potassium phosphate (along with high concentrations of sulfhydryl reductant) for dissolving the ammonium sulfate pellets was effective in preventing aggregation. It is essential to maintain titin's sulfhydryls in a reduced state; once aggregates were formed it was impossible to convert them back to monomers even with high concentrations of sulfhydryl reductant (i.e., 10 mM DTT) (unpublished observations).

Our results indicate that native bovine cardiac titin consists primarily of a mixture of β-sheet (53%) and random coil (47%) at neutral pH as determined from circular dichroism measurements. Previous studies using chicken skeletal muscle β-connectin (titin T2) and porcine cardiac β-connectin suggested the presence of at least 60% β-sheet in the secondary structures (Maruyama et al., 1986; Itoh et al., 1986). The slightly lower β-sheet content of bovine cardiac titin may indicate there is less β structure in the protein segment which is missing from the titin T2. However, this discrepancy might also be due to different methods of calculating secondary structure parameters, different equipment, and different animal species. Furthermore, the current work has employed more dilute titin solutions (0.1–0.25 mg/mL) to prevent protein aggregation and has included 1 mM DDT to eliminate possible disulfide bond formation. The high β-sheet content and the absence of α-helix content are consistent with a model of titin composed primarily of immunoglobulin C2 and fibronectin type III motifs throughout most of its length, since both types of motifs contain seven-stranded β-sheets as their dominant secondary structure (Leahy et al., 1992; Holden et al., 1992).

It has been known that titin has a tendency to aggregate, and high salt and alkaline pH are required to keep titin molecules dissociated (Wang et al., 1984; Trinick et al., 1984; Maruyama et al., 1984). There is evidence that polypeptide aggregates may display a conformation quite different from the structures of the nonaggregated polypeptides under identical conditions (Hammes & Schullery, 1968). In the

present study, the effects of ionic strength and pH were investigated by circular dichroism spectroscopy. Our results showed that increasing the ionic strength between 0.2 and 0.6 M had negligible influence upon the secondary structure of bovine cardiac titin.

Previous work on various SDS–protein complexes has shown them to be mixtures of α-helix and random coil, with the amount of helix present ranging up to about 40% (Greenfield & Fasman, 1969; Mattice et al., 1976). Our results were consistent with the above findings and indicated that SDS–titin complexes contained 35% α-helix and 65% random coil. The finding of significant increases in α-helix content of titin T1 in the presence of SDS confirms similar results obtained using Raman spectroscopy of β-connectin (Uchida et al., 1991).

The biological function of titin in muscle requires further study. Pure native full-length cardiac titin isolated by our method should prove useful for future protein-binding and filament-assembly studies.

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