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Isolation of a Terminal Cisterna Protein Which May Link the Dihydropyridine Receptor to the Junctional Foot Protein in Skeletal Muscle†

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Received April 27, 1990; Revised Manuscript Received June 27, 1990

ABSTRACT: The isolated dihydropyridine receptor and junctional foot protein were employed as protein ligands in overlay experiments to investigate the mode of interaction of these two proteins. As previously demonstrated by Brandt et al. [Brandt et al. (1990) *J. Membr. Biol. 113*, 237–251], the DHP receptor directly binds to an intrinsic terminal cisterna protein of M_r 95 000 (95-kDa protein). The junctional foot protein also binds to an M_r 95 000 protein showing similar organelle distribution to the 95-kDa protein which binds to the dihydropyridine receptor. The 95-kDa protein which binds to the dihydropyridine receptor was isolated to over 85% purity employing sequential column chromatography. Junctional foot protein and dihydropyridine receptor overlays of the column fractions at successive stages of isolation show an identical pattern of distribution, indicating that both probes bind to the same protein. When CHAPS-solubilized terminal cisterna/triads were passed through Sepharose with attached 95-kDa protein, the junctional foot protein was specifically retained, as evidenced by ryanodine binding. The junctional foot protein was incompletely released by 1 M NaCl. The α_1 subunit but not the β subunit of the dihydropyridine receptor was also specifically retained, as evidenced by immunoblotting employing dihydropyridine receptor subunit-specific antibodies. A 170-kDa Stains-all blue staining protein, which appears to be bound to the luminal side of the terminal cisterna, was also retained on the 95-kDa protein column. From these findings, a model for the triad junction is proposed.

One major hypothesis for excitation–contraction (EC) coupling, as originally proposed by Schneider and Chandler (1973), states that the voltage sensor molecules in the transverse (T)-tubule undergo a conformational change that is transmitted through the feet of the terminal cisternae (TC) abutting the T-tubule to activate the sarcoplasmic reticulum (SR) Ca^{2+} release. The junctional foot protein (JFP) was originally identified as a high molecular weight doublet by Cadwell and Caswell (1982) and later isolated and localized to the triad junction by immunoelectron microscopy (Kawamoto et al., 1986). Subsequent biochemical and electrophysiological studies indicated that the JFP is the ryanodine-sensitive Ca^{2+} release channel of the SR (Imagawa et al., 1987; Hymel et al., 1987; Lai et al., 1988). The dihydropyridine (DHP) receptor is present at unusually high density in the T-tubules (Fosset et al., 1983; Glossman et al., 1983), but only a few percent of the DHP receptor appear to function as Ca^{2+} channels (Schwartz et al., 1985). Rios and Brum (1987) have proposed that the DHP receptor is the voltage sensor of the T-tubule. Tanabe et al. (1988) have supported this proposal in studies of dysgenic mice which exhibit EC coupling only after introduction of cDNA for the α_1 subunit of the DHP receptor. On the basis of these findings, several models have been proposed in which the DHP receptor and JFP bind directly to each other. Conformational changes of

† This work was supported by a Lucille P. Markey fellowship, by NIH Grants AR21601 and HL36029, and by grants from the Muscular Dystrophy Association and the American Heart Association, Florida affiliate.
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0006-2960/90/0429-9281$02.50/0

the DHP receptor may thereby modulate the opening of the SR Ca^{2+} release channel directly (Block et al., 1988; Hymel et al., 1989; Takeshima et al., 1989). However, there has as yet been no demonstration of a direct association between the DHP receptor and the JFP, raising the possibility that an intermediary protein may participate in the interaction.

Recently, our laboratory has identified a TC protein of M_r 95 000 (95-kDa protein) which specifically binds to the DHP receptor in Western blot overlay experiments (Brandt et al., 1990). Detergent and high salt treatments have indicated that the 95-kDa protein is an intrinsic protein of the junctional complex of the TC. The 95-kDa protein was enriched in the strong triads which have been recently identified as a subpopulation of triads showing unusual resistance to conventional breakage procedures. These triads exhibit thin filamentous connections between the T-tubules and TC with relatively infrequent feet in the junction (Kim et al., 1990).

In this report, we demonstrate that the purified JFP also binds to the 95-kDa protein. We have isolated the 95-kDa protein using column chromatographic procedures and employed it to demonstrate the specificity of interaction between the 95-kDa protein and other triadic proteins. A model of the protein–protein interactions of the triad junction is proposed.

Materials and Methods

Preparation of Membranes. TC/triads were isolated from rabbit skeletal muscle as described by Caswell et al. (1976). T-Tubules, light terminal cisternae (LTC), and heavy terminal cisternae (HTC) were prepared by French press treatment of the TC/triads as described by Brandt et al. (1980). Strong triads were isolated from the LTC as described by Kim et al. (1990). On the basis of assay of DHP receptor content, the strong triads represent one-third to half of the original triadic preparation. Protein was determined according to Bradford (1976). When the protein sample was in a medium containing detergent, BCA reagent (Pierce) was used. Bovine serum albumin was employed as standard.

Isolation and Labeling of Proteins. JFP was isolated from CHAPS-solubilized TC/triads as described previously (Brandt et al., 1990) except that iodination was performed on the eluate of the hydroxyapatite column employing Iodobeads (Pierce). The sample was then applied to the heparin–agarose column to complete the purification and to remove unreacted $^{125}I_2$ and $^{125}I^-$. The DHP receptor was isolated from TC/triads according to protocols described by Talvenheimo et al. (1987) and radiolabeled as previously described (Brandt et al., 1990) except that Iodobeads (Pierce) were employed. Biotinylation of the DHP receptor was carried out by using the eluate from the DEAE-Sephadex column as described above except that 10 mM NaP_i replaced 25 mM Tris-HCl. The DHP receptor (100–200 μg) was mixed with less than 1 mg of sulfo-NHS-biotin (Pierce) and incubated for 30 min on ice. Residual sulfo-NHS-biotin was inactivated by reaction with 10 mM Tris-HCl, pH 7.4. The reaction mixture was applied to a 3-mL Sephadex G-50 column preequilibrated with 20 mM potassium gluconate, 20 mM MOPS–Tris, pH 7.4, and 40 mM NaCl and precentrifuged at 1000*g* for 30 s. Sample-loaded columns were centrifuged at 1000*g* for 2 min, and the eluate was used directly for overlay assays.

Preparation of the DHP Receptor Subunit-Specific Antibodies. Subunit-specific antibodies were prepared by affinity purification from polyclonal antiserum collected from mice immunized with DHP receptor partially purified by WGA–Sepharose chromatography as described by Talvenheimo et al. (1987). DHP receptor subunits were separated by SDS–PAGE on an 8% separating gel under reducing conditions and then electroblotted onto a nitrocellulose membrane. Strips of nitrocellulose corresponding to the α_1 and β subunits were cut out and incubated overnight with 3% bovine serum albumin (BSA), 150 mM NaCl, and 25 mM Tris-HCl, pH 7.4, and then incubated for 3 h at 4 °C with the polyclonal antiserum. The strips were washed 3 times with 0.5% BSA, 0.05% Tween-20, 150 mM NaCl, and 25 mM Tris-HCl, pH 7.4, and antibodies were eluted by acidic pH treatment (Olmsted, 1981). Both the anti-α_1 and anti-β DHP receptor subunit-specific antibodies immunoprecipitate the intact DHP receptor as assayed by ligand binding.

Electrophoresis and Western Blotting. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and Western blotting were carried out as described by Brandt et al. (1990) with the following exceptions: (1) 40 mM NaCl was included in the incubation and wash media of the blots; (2) when JFP was employed as a probe, the incubation medium contained 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfate (CHAPS) and 0.1% egg phosphatidylcholine (PC); and (3) biotinylated DHP receptor was assayed by streptavidin–horseradish peroxidase conjugate catalyzed reaction using 4-chloro-1-naphthol. The immunoblots employing the anti-α_1 and β DHP receptor subunit-specific antibodies were developed by employing anti-mouse IgG–horseradish peroxidase conjugate and 4-chloro-1-naphthol as a substrate.

Stains-all staining was carried out according to the procedures of Campbell et al. (1983). In some experiments, Coomassie blue stained gel lanes were scanned with a Zeineh Model SL-TRFF scanning densitometer. Protein peak areas (hatched areas) were estimated by cutting and weighing.

Isolation of the 95-kDa Protein. TC/triads were washed with 1 M KCl and diluted to 1 mg of protein/mL (approximately 70–100 mL) in a medium of 1% Lubrol PX, 1 M KCl, 5 mM Tris-HCl, 0.1% PC, and 30 mM KP_i, pH 7.4. This was centrifuged for 1 h at 120000*g*, and the supernatant was passed through a hydroxyapatite (HA) column (25-mL packed bed volume) preequilibrated with 30 mM KP_i, 970 mM KCl, 0.2% polyoxyethylene(9) lauryl ether (C12E9), 0.02% PC, and 5 mM Tris-HCl, pH 7.4. The HA colum was washed with 1 column volume of the buffer above and sequentially eluted with a medium containing 100 mM KP_i, pH 7.4, 900 mM KCl, 0.2% C12E9, 0.02% PC, and 5 mM imidazole followed by the above medium but containing 250 mM KP_i and 750 mM KCl. The 250 mM KP_i eluate was passed directly from the HA column onto an imidodiacetate–Sepharose 4B column charged with cupric ion (2-mL bed volume) prepared by the method of Porath and Olin (1983). The metal column was preequilibrated with 30 mM NaP_i, pH 7.4, 1 M NaCl, 5 mM imidazole, 0.2% C12E9, and 0.02% PC. The output of this column was directly connected to a Sephacryl S-500 HR column (1.5 × 60 cm) which was preequilibrated with the same medium in which 5 mM imidazole was replaced with 5 mM Tris-HCl. The fractions close to the void volume containing the 95-kDa protein were passed onto a small HA column (0.5-mL bed volume) preequilibrated with S-500 column elution buffer and washed with 30 mM KP_i, pH 7.4, 970 mM KCl, 0.2% C12E9, 0.02% PC, and 5 mM Tris-HCl, pH 7.4. The column was subsequently eluted stepwise with the above medium but containing 100 mM KP_i, pH 7.4, and 900 mM KCl followed by the above medium containing 400 mM KP_i, pH 7.4, and 600 mM KCl. All media employed during the isolation contained the following protease inhibitors: 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 μM leupeptin, and 1 μM pepstatin A, and isolation was carried out at 0 °C.

95-kDa Protein Affinity Chromatography. The 95-kDa protein was prepared as described above with omission of 5 mM Tris-HCl during the second small HA column chromatography. The eluates from the second HA column were coupled to 0.5 mL of CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions (sample column). Control columns lacking 95-kDa protein were prepared identically except that an equal volume of phosphate elution medium was coupled to CNBr-activated Sepharose. When solubilized TC/triads were employed, the columns were preequilibrated with 10 mM MOPS–Tris, pH 7.4, 60 mM NaCl, 0.2% CHAPS, and 0.02% PC containing protease inhibitors. When phosphorylated T-tubules were used, the medium contained 40 mM NaCl and 20 mM NaF instead of 60 mM NaCl.

TC/triads (20 mg in 4.4 mL of 35% sucrose) were incubated with 10 nM [^{3}H]ryanodine (2 μCi) in the presence of 10 mM MOPS–Tris, pH 7.4, 1 mM ATP, 0.8 M NaCl, and protease inhibitors for 1 h at room temperature with stirring. The vesicles were diluted with ryanodine-free incubation medium and pelleted at 120000*g* for 1 h. The pellet was resuspended in 250 mM sucrose/2 mM histidine, pH 7.4, to 7 mg/mL and solubilized by adding 1.4% CHAPS, 0.14% PC, and 0.8 M NaCl. Undissolved material was removed by centrifugation in an Airfuge (Beckman) at 30 psi for 15 min. The supernatant was diluted 1:13 with 10 mM MOPS–Tris, pH 7.4, 0.2% CHAPS, and 0.02% PC to a final concentration of 60 mM NaCl and applied to the sample and control columns. The columns were washed with 8 mL of the above medium (containing 60 mM NaCl) and subsequently eluted with 10 mM MOPS–Tris, pH 7.4, 1 M NaCl, 0.2% CHAPS, and 0.02% PC followed by 10 mM MOPS–Tris, pH 7.4, and 1% SDS. Aliquots (100 μL) from individual fractions were counted for [^{3}H]ryanodine in toluene–Triton-based scintillation cocktail and assayed by SDS–PAGE and immunoblotting using anti-DHP receptor antibodies. In the case of T-tubules, 16 mg of T-tubules was phosphorylated in 1 mL of medium containing 20 mM MOPS–Tris, pH 7.0, 2 mM $MgCl_2$, 2 mM EDTA, 25 mM NaF, 0.2 unit of catalytic subunit of cAMP-dependent protein kinase, and 90 μM [^{32}P]ATP (10^4 dpm/pmol) for 2 min at room temperature. The phosphorylated T-tubules were mixed with 3 mL of ice-cold 50 mM NaF, 50 mM NaP_i, pH 7.0, and 20 mM EDTA, pelleted, resuspended in 250 mM sucrose, 2 mM histidine, and 20 mM NaF, and processed further as described above with the exception that 20 mM NaF was included in the media while NaCl concentration was reduced to 40 mM. All the chromatographic procedures were carried out at 0 °C, and the media contained the protease inhibitors described earlier.

Materials. Hydroxyapatite, imidodiacetate–Sepharose 6B, DEAE-Sephadex, catalytic subunit of cAMP-dependent protein kinase, C12E9, Lubrol PX, CHAPS and egg phosphatidylcholine were purchased from Sigma. Sephacryl S-500 HR was from Pharmacia. Iodobeads, BCA reagent, and sulfo-NHS-biotin were from Pierce. Na^{125}I and [^{3}H]ryanodine were from New England Nuclear. Streptavidin–horseradish peroxidase conjugate was from Amersham Corp. Anti-mouse antibodies–horseradish peroxidase conjugate was from Hyclone.

Results

Interaction of the JFP with the 95-kDa Protein. Recently, we reported the interations of the DHP receptor with microsomal membrane proteins (Brandt et al., 1990). The DHP receptor bound an intrinsic protein of M_r 95000 which is localized to the junctional region of the TC. In Figure 1, we have extended these findings using purified JFP as a ligand for Western blot overlay. Panel A shows the Coomassie blue stained proteins from the isolated membrane fractions, and panel B shows the autoradiogram of these fractions blotted and overlaid with purified ^{125}I-JFP. Panel C is the autoradiogram of the JFP employed in these experiments. In longitudinal reticulum (lane 1), JFP binds only to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) at 36 kDa. In T-tubules (lane 2), JFP similarly binds to GAPDH as described previously (Brandt et al., 1990). In contrast, the JFP intensely labeled a M_r 95000 protein underlying the major SR protein, Ca^{2+}-ATPase in the LTC (lane 3), HTC (lane 4), and strong triads (lane 6) with a weaker but significant activity in TC/triads (lane 5). The intensity of labeling of the 95-kDa protein (panel B) is in the same order as the content of JFP in the vesicles (panel A, lanes 1 and 3–6). The slightly different mobility of the radiolabeled 95-kDa protein in lanes 3–6 is probably due to the different amount of the Ca^{2+}-ATPase pushing the 95-kDa protein downward. Additional labeling at low intensity on 80- and 60-kDa proteins was observed. Binding also occurred to the glycolytic enzymes aldolase and GAPDH while binding to the DHP receptor or to calsequestrin was not observed. The protein of M_r 95000 here labeled with the JFP has a similar molecular mass and organelle distribution to the M_r 95000 protein which was previously identified by the DHP receptor overlay (Brandt et al., 1990). To test whether these two proteins are identical, the 95-kDa protein was isolated.

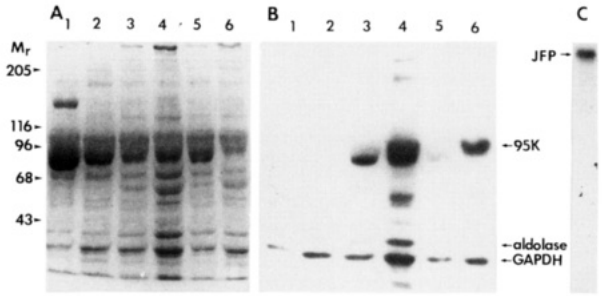

FIGURE 1: Overlay of subcellular organelles with ^{125}I-JFP. Organelles were electrophoresed in duplicate on a 5.5% separating gel. One set was stained with Coomassie blue to show the protein composition of various membranes (panel A). The other set was electroblotted onto a nitrocellulose membrane, overlaid with ^{125}I-JFP, and autoradiographed (panel B). Panel C shows the autoradiogram of the purified JFP. For panels A and B: lane 1, longitudinal reticulum (25 μg); lane 2, transverse tubule (25 μg); lane 3, light TC (25 μg); lane 4, heavy TC (25 μg); lane 5, TC/triads (25 μg); lane 6, strong triads (20 μg). The protocols for membrane isolation, electrophoresis, electroblotting, and overlay are detailed under Materials and Methods. JFP was purified from CHAPS-solubilized TC/triads and iodinated with Iodobeads (Pierce) as described under Materials and Methods. The molecular weights of molecular standards (Sigma) are indicated on the left ($\times 10^{-3}$).

Isolation of the 95-kDa Protein. The fate of the 95-kDa protein during each chromatographic step was monitored by DHP receptor overlay. Lubrol PX solubilized the 95-kDa protein better than CHAPS and digitonin in the presence of 1 M KCl. The 95-kDa protein precipitated in low ionic strength medium, and, therefore, the total salt concentration was kept at 1 M throughout the isolation procedure.

(*A*) *HA Chromatography.* The UV absorbance trace of the output from the hydroxyapatite (HA) column sequentially eluted with increasing P_i is shown in Figure 2A. Figure 2B shows a Coomassie blue stained gel, and Figure 2C is the overlay by biotinylated DHP receptor of column fractions marked in panel A. Lane B1 shows the intact TC/triads indicating the presence of the 95-kDa protein (lane C1). The

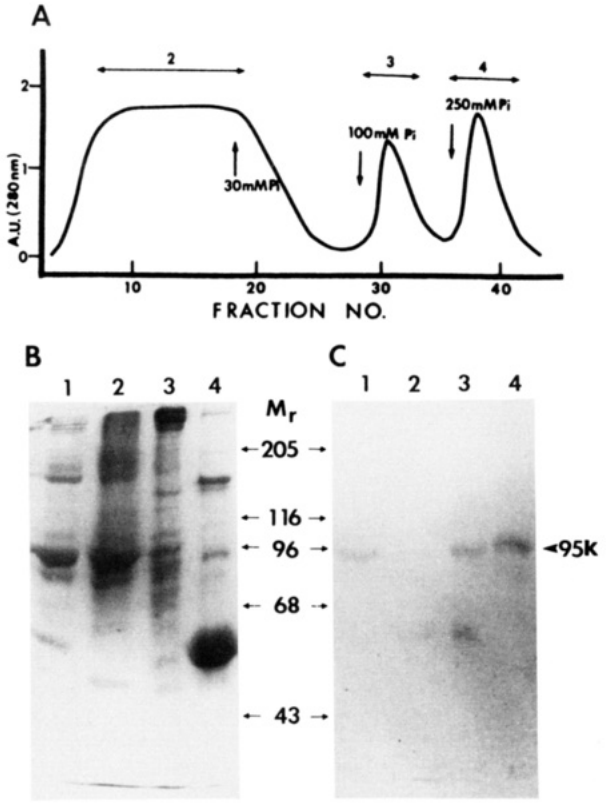

FIGURE 2: Hydroxyapatite (HA) chromatography of salt-washed TC/triads. TC/triads were washed with 1 M KCl, solubilized, and chromatographed on an HA column as described under Materials and Methods. HA fractions were electrophoresed in duplicate and either stained with Coomassie blue or electroblotted for the biotinylated DHP receptor overlay. (Panel A) UV absorbance record of output from the HA column. Addition of elution media is indicated along with the fractions analyzed by SDS–PAGE. (Panel B) Coomassie blue stained gel of column fractions in panel A. (Panel C) Horseradish peroxidase stained blot overlaid with the biotinylated DHP receptor. For panels B and C: lane 1, TC/triads (25 μg); lane 2, flow through (50 μL); lane 3, 100 mM KP_i eluate (50 μL); lane 4, 250 mM KP_i eluate (50 μL). Molecular weight standards are indicated $\times 10^{-3}$.

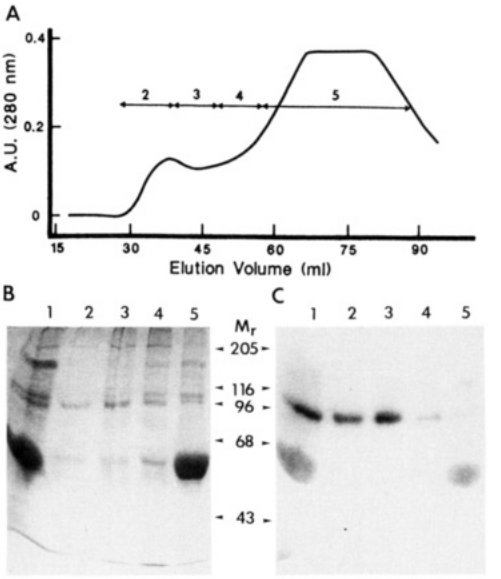

FIGURE 3: Sephacryl S-500 chromatography of the 250 mM KP_i eluate from the HA column. The 250 mM eluate from HA column was sequentially passed through a copper chelate column and Sephacryl S-500 HR column (S-500), which were connected in tandem. Aliquots (100 μL) of the S-500 output were electrophoresed in duplicate and either stained with Coomassie blue or electroblotted for subsequent overlay with ^{125}I-DHP receptor as described under Materials and Methods. (Panel A) UV absorbance record of the output from the S-500 column. (Panel B) Coomassie blue stained gel. (Panel C) Autoradiogram of the blot overlaid with the ^{125}I-DHP receptor. For panels B and C: lane 1, 250 mM eluate from the HA column (50 μL); lanes 2–5, 100-μL aliquots of pooled fractions indicated in panel C. Molecular weight standards are indicated $\times 10^{-3}$.

flow-through fraction in the presence of 30 mM KP_i contains Ca^{2+}-ATPase and other proteins (lane B2) but does not contain the 95-kDa protein (lane C2). The 100 mM KP_i step, which was discarded (lane B3), eluted most of the JFP, residual Ca^{2+}-ATPase, and some 95-kDa protein (lane C3). The 250 mM KP_i step (lane B4) eluted major proteins at 170, 95, and 63 kDa (calsequestrin). This included the 95-kDa protein detected by the DHP receptor overlay (lane C4).

(B) Tandem Copper Chelate–Sephacryl S-500 Column Chromatography. The 250 mM P_i eluate from the HA column was passed through a copper chelate column followed by a Sephacryl S-500 HR (S-500) molecular sieve column coupled in tandem. A preliminary study demonstrated that the 95-kDa protein was excluded from the copper chelate column preequilibrated with 5 mM imidazole while some of the higher molecular weight peptides remained bound. The UV absorbance of the output from the S-500 column is shown in Figure 3A. Figure 3B shows a Coomassie blue stained gel, and Figure 3C shows the overlay by ^{125}I-DHP receptor of fractions marked in Figure 3A. Lane B1 shows the 250 mM KP_i eluate from the previous HA column indicating the presence of the 95-kDa protein (lane C1). The weak labeling discernible in the position of calsequestrin is probably nonspecific and reflects the high concentration of calsequestrin present in the eluate at this stage of the purification. Two UV-absorbing peaks were eluted from the S-500 column: one at the void volume and the other close to the total volume. The 95-kDa protein eluted close to the void volume (lanes 2 and 3) while calsequestrin eluted close to the total column volume (lane 5), effectively separating these two proteins. Additional minor proteins including one of similar molecular weight to that of the 95-kDa protein but not reacting with DHP receptor are observed in the gel of eluates from the column.

(C) Second HA Chromatography. The M_r 95 000 protein peak was readsorbed onto a small HPT column for concentration and further purification. The column was subsequently eluted stepwise with 100 mM KP_i and 400 mM KP_i. Figure 4 shows the protein patterns of those eluates and corresponding densitometric traces. The 100 mM KP_i eluate (panel A) shows a single protein band at 95 kDa of at least 87% purity. The purity of the 400 mM KP_i eluate (panel B) was estimated as 65% owing to contamination with a 205-kDa protein.

Identity of the 95-kDa Protein to the Protein Which Binds to the JFP. The DHP receptor and JFP might both bind to an identical protein or to two distinct proteins with similar molecular weight. To test these possibilities, blots of selected fractions during 95-kDa protein purification were probed with JFP. Figure 5A shows the Coomassie blue stained gel, and Figure 5B shows the autoradiogram of the corresponding blot. Lane A1 is intact TC/triads showing the presence of a protein of M_r 95 000 revealed by JFP overlay (lane B1). Lanes A2 and A3 respectively are the flow-through and 100 mM KP_i eluates from the HA column which are low in JFP binding protein (lanes B2 and B3). These fractions were discarded according to protocols in Figure 2 in order to minimize Ca^{2+}-ATPase contamination. The low content of JFP overlain protein in the flow-through fraction corresponds to a low content of 95-kDa protein shown in Figure 2. In a number of preparations, some variability in the content of the 95-kDa protein in the 100 mM KP_i eluate from the HA column has

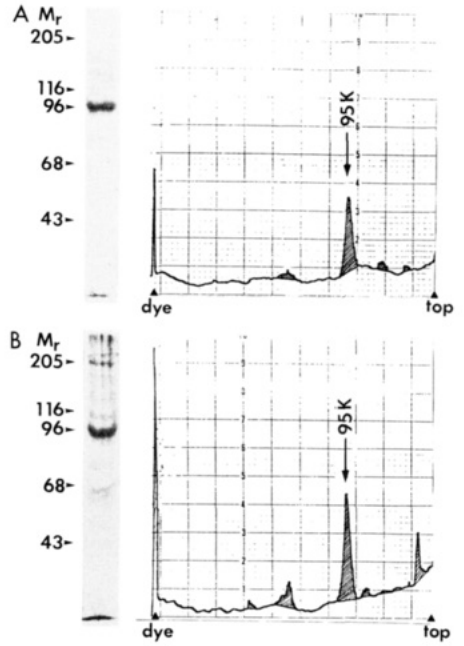

FIGURE 4: Densitometric scan of the purified 95-kDa protein. The peak fractions containing the 95-kDa protein were readsorbed onto a small HA column and eluted with a medium containing 100 mM KP_i followed by a medium containing 400 mM KP_i as described under Materials and Methods. (Panel A) Coomassie blue stained gel of 100 mM KP_i eluate (3 μg) from the small HA column and its densitometric trace. (Panel B) Coomassie blue stained gel of 400 mM KP_i eluate (5 μg) and its densitometric trace. Molecular weight standards ($\times 10^{-3}$) are indicated on the left.

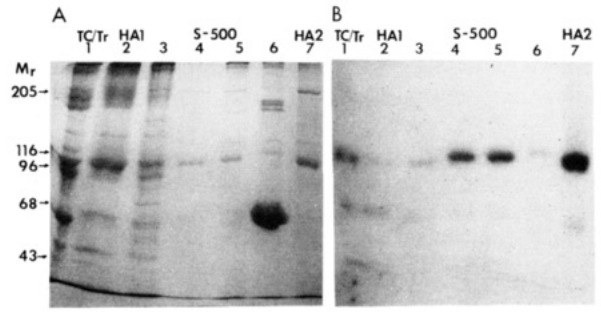

FIGURE 5: Overlay of various fractions during purification of the 95-kDa protein with ^{125}I-JFP. Purification of the 95-kDa protein was carried out as described in Figures 2–4 with two exceptions: (1) the 250 mM KP_i eluate from the HA column was directly loaded onto the copper chelate–S-500 column; (2) the 95-kDa protein from the small HA column was eluted with a single step of 400 mM KP_i. (Panel A) Coomassie blue stained gel. (Panel B) Autoradiogram of the electroblotted fractions overlaid with the purified ^{125}I-JFP. For panels A and B: lane 1, TC/triads (25 μg); lane 2, flow-through fraction from the first HA column (50 μL); lane 3, 100 mM KP_i eluate from the first HA column (50 μL); lanes 4–6 are from the S-500 column (100 μL/lane); lane 4, pooled fractions of the first peak eluted close to the void volume; lane 5, pooled intermediate fractions between first and second peaks; lane 6, pooled fractions of the second major peak; lane 7,400 mM KP_i eluate from the small HA column (5 μg). Overlay and iodination of the isolated JFP were carried out as described under Materials and Methods. Molecular weight standards are indicated on the left ($\times 10^{-3}$).

been discerned by using both probes. The 250 mM KP_i eluate from the HA column is not shown in this figure since it was passed directly onto the next column. Lanes 4–6 represent the output from the molecular sieve column. Lanes 4 and 5 are close to the void volume and lane 6 close to the total column volume. A clear protein band at M_r 95 000 seen in lanes 4 and 5 shows corresponding high activity of JFP overlay (lanes B4 and B5) while little activity is observed in the fraction in which calsequestrin elutes (lane B6). This mimics very closely to the observations of Figure 3. Lane 7 shows the 400 mM KP_i eluate from the second HA column employed to concentrate the 95-kDa protein. The heavy labeling with JFP shows that we have similarly concentrated the JFP binding protein.

95-kDa Protein Affinity Chromatography. The previous experiments established that the 95-kDa protein binds to the DHP receptor and the JFP but do not address the specificity of binding of the 95-kDa protein. Purified 95-kDa protein was covalently attached to a matrix support and employed as an affinity column to extract proteins which bind to it. CHAPS-solubilized TC/triads prelabeled with [^{3}H]ryanodine were passed through the column. A second aliquot of solubilized triads was passed through a CNBr-activated Sepharose column without attached 95-kDa protein (control column). Figure 6A shows the profiles of [^{3}H]ryanodine eluted from each column. Most of the [^{3}H]ryanodine passed directly through both sample and control columns. However, a distinct peak was subsequently eluted from the 95-kDa protein–Sepharose column by 1 M NaCl but not from the control column. Treatment with SDS released additional [^{3}H]ryanodine in a sharper and larger peak than that produced by hypertonic salt from the sample column. A negligible amount of [^{3}H]ryanodine was eluted from the control column. Figure 6B shows SDS–PAGE of fractions marked in Figure 6A. Lane 1 is intact TC/triads. Lane 2 is the flow-through fraction from the column which contains some JFP discernible almost at the top of the gel. Lanes 3–5 shows NaCl eluates from the 95-kDa protein column while lanes 8–10 are corresponding fractions from the control column. Comparison of sample and control columns shows the presence of proteins bound nonspecifically to the gel matrix at M_r 90K–110K. Specifically bound proteins present in the 95-kDa protein column but not in the control column include JFP (arrowhead) and a 170-kDa protein (asterisk). Lanes 6 and 7 are SDS eluates from the 95-kDa protein column while lane 11 is the corresponding fraction from the control column. Again, some nonspecifically bound proteins are eluted from both columns including M_r 180K and 90K–110K. JFP is eluted from the 95-kDa protein column but not from the control column. These data indicate that the binding of JFP to the 95-kDa protein is sufficiently strong that even 1 M NaCl does not completely dissociate the 95-kDa protein–JFP complex during the time of interaction. Furthermore, the presence of ryanodine in this tightly bound fraction indicates that at least some of the tetrameric form of JFP retained.

We tested whether the retained protein of M_r 170K was the α_1 subunit of the DHP receptor. Hamilton et al. (1989) reported that CHAPS detergent caused the DHP receptor to dissociate into its subunits but this treatment also caused ligand dissociation. Therefore, we employed affinity-purified anti-α_1 and β subunit antibodies (Figure 6C). Lane 1 is the 1 M NaCl eluate from the 95-kDa protein column electrophoresed, blotted, and incubated with the anti-α_1 and β subunit antibodies, showing a positive reaction at the region of 170 kDa but not at the position of the β subunit. The 170-kDa immunoreactive band appeared as a doublet; this has been seen in some but not all of our DHP receptor preparations. Lanes 2–4 define the specificity of the antibodies. Lanes 3 and 4 show immunoblots of partially purified DHP receptor (lane 2, Amido-black stain) incubated with anti-α_1 and β subunit antibodies, respectively.

Stains-all staining (Figure 6D) of the gel of Figure 6B shows three blue-staining proteins in TC/triads (lane 1). An intense band at 170 kDa is visible with a fainter band at 160 kDa

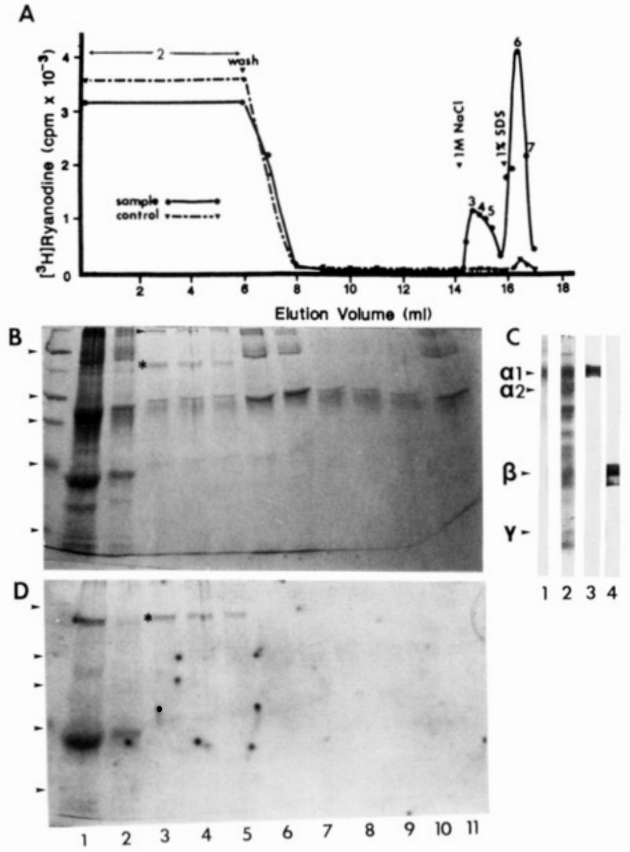

FIGURE 6: 95-kDa protein affinity chromatography of CHAPS-solubilized TC/triads. The sample column contained the 400 mM KP_i eluate from the small HA column (200 μg). The control column was prepared by coupling the 400 mM KP_i elution medium. Twelve milligrams of TC/triads was labeled with [^{3}H]ryanodine, solubilized, and applied to sample and control columns. (Panel A) Profiles of [^{3}H]ryanodine activity from the sample and control columns. Addition of the wash, 1 M salt, and 1% SDS buffer are indicated along with the fractions analyzed by SDS–PAGE. (Panel B) Coomassie blue stained gel showing protein composition of eluates from the sample and control columns. (Panel C) Immunoblot of the 1 M NaCl eluate from the sample column employing the affinity-purified DHP receptor subunit-specific antibodies. (Panel D) Stains-all stained gel of panel B. For panels B and D: lane 1, TC/triad (20 μg); lane 2, flow through from sample column; lanes 3–5, sequential 1 M NaCl eluates from sample column; lanes 6–7, SDS eluates from sample column; lanes 8–10, 1 M NaCl eluates from control column; lane 11, SDS eluate from control column. Lanes 2–11 were loaded with 100-μL aliquots of individual fractions. Molecular weights of standards are indicated on the left ($\times 10^{-3}$). In lane 3, an arrowhead and asterisk indicate the JFP and the 170-kDa protein, respectively. For panel C: lane 1, immunoblot of pooled fractions of NaCl eluate from sample column (100 μL of pooled fractions 3, 4, and 5 in panel B) incubated with combined α_1 and β DHP receptor subunit-specific antibodies; lane 2, Amido-black staining of electroblotted DHP receptor partially purified by WGA–Sepharose chromatography; lanes 3 and 4, immunoblots of lane 2 incubated with anti-α_1 subunit antibody (lane 3) and anti-β subunit antibody (lane 4). For the Western blot of the 1 M NaCl eluate from the 95-kDa affinity column, the anti-DHP receptor antibodies with the same dilution factor as in the Western blot of DHP receptor were employed. Four subunits of the DHP receptor are indicated on the left (panel C). For Stains-all staining, the Coomassie blue stained gel was destained with 25% isopropyl alcohol and then stained with Stains-all following the procedures described under Materials and Methods. Molecular weight standards indicated on the left ($\times 10^{-3}$) are the same as in Figure 1.

visible just below. The major lower molecular weight band is calsequestrin. In the flow-through fraction from the sample column (lane 2), the 160-kDa protein running below the 170-kDa protein of lane 1 is present together with calsequestrin. The band eluted by 1 M NaCl from the sample column (D3–D5) at the molecular weight of the upper band

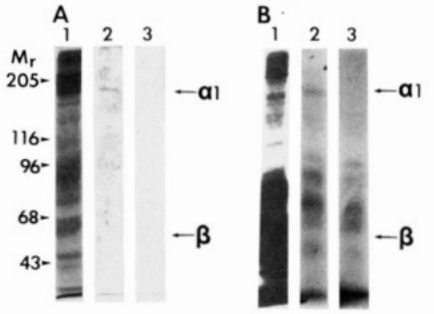

FIGURE 7: 95-kDa protein affinity chromatography of T-tubules. The sample column was prepared by coupling the 95-kDa protein (400 mM KP_i eluate from the small HA column, 150 μg) as in Figure 6. T-tubules (9 mg) were phosphorylated using [^{32}P]ATP (10^4 dpm/pmol) with the catalytic subunit of cAMP-dependent protein kinase, solubilized with CHAPS, and applied to the sample and control columns as described under Materials and Methods. The columns were washed and eluted identically as in Figure 6. (Panel A) Coomassie blue stained gel. (Panel B) Autoradiogram of the gel of panel A: lane 1, T-tubules (20 μg); lane 2, 1 M NaCl eluate from sample column (100 μL of pooled fractions); lane 3, 1 M NaCl eluate from control column (100 μL of pooled fractions). The positions of the α_1 and β subunits of DHP receptor are indicated on the right. Molecular weight markers ($\times 10^{-3}$) are the same as in Figure 1.

(170 kDa) of lane 1 stains blue with Stains-all. A positive Stains-all reaction is not a characteristic of the DHP receptor subunits. Two additional Stains-all blue proteins, 160-kDa protein and calsequestrin, were not retained on the column. Therefore, the 170-kDa protein band specifically retained on the sample column consists of at least two different proteins, the α_1 subunit of DHP receptor and the 170-kDa Stains-all blue protein.

The identification of the α_1 subunit of the DHP receptor has been confirmed by repeating the 95-kDa protein affinity chromatography employing T-tubules in which the α_1 and β subunits were labeled by phosphorylation with protein kinase A (Figure 7). The T-tubular fraction does not contain Stains-all blue staining 170-kDa protein. Figure 7A shows a Coomassie blue stained gel, and Figure 7B shows the autoradiogram of that gel. Lane 1 is the T-tubule. Lane 2 is the 1 M NaCl eluate from the 95-kDa protein column, and lane 3 is the eluate from the control column. A single protein at M_r 170K detectable by Coomassie blue staining is present in the eluate from sample (lane A2) but not from control column (lane A3). The autoradiogram shows that this band is phosphorylated (B2). In addition, a small portion of heavily phosphorylated proteins is nonspecifically retained by CNBr-activated Sepharose. Phosphoprotein in the molecular weight range of the β subunit of DHP receptor was not observed.

Discussion

In order to propose molecular mechanisms for EC coupling in skeletal muscle, it is necessary to define the protein–protein interactions of those protein moieties established to be involved in the signaling process. We previously reported that the DHP receptor in the T-tubule and the JFP in the TC did not directly interact but that these two proteins could form a ternary complex through GAPDH or aldolase. We also observed that the DHP receptor bound to a previously unknown M_r 95 000 protein on the TC membrane and that interaction occurred in hypertonic saline. We have employed two technical approaches, protein overlay and affinity chromatography, to investigate these interactions. Each technique in itself has both advantages and limitations, but the two approaches are to a considerable extent complementary and confirmatory. The protein overlay technique detects only direct protein–protein

interactions as apposed to interactions through a third protein. Such interactions might not be observed in solution because one protein may not be soluble in the conditions in which interactions may be expected to occur, e.g., low ionic strength. This technique involves denaturation and subsequent renaturation of proteins, and hence proteins on the blot may not completely renature into their native state and thereby be inactivated. However, there is considerable evidence that proteins frequently renature, as evidenced by retention of enzymatic activity (Sock & Rohringer, 1988), Ca^{2+} binding (Zorzato & Volpe, 1988), and plasma lipoprotein binding (Hofmann et al., 1989) of Western-blotted proteins. The blotting technique may artifactually expose proteins or sites normally inaccessible, giving rise to interactions which might not occur in vivo in which these two proteins would normally be separated by a membrane, e.g., calsequestrin binding to GAPDH (Brandt et al., 1990).

Affinity chromatography is carried out on proteins dissolved using nondenaturing detergents and hence avoids the problem of loss of binding activity. This technique detects indirect as well as direct interactions if several proteins are passed simultaneously through the column but does not give the order of the complex. The method can only be employed successfully if it is possible to dissolve and chromatograph the proteins under conditions in which binding is expected to occur, e.g., low ionic strength. Affinity chromatography allows observation of interactions which cannot be observed in free solution when one of the proteins is poorly soluble since this protein can be immobilized on the gel. However, because the liganding proteins are solubilized in detergent, interactions may occur which do not take place in vivo owing to a membrane barrier.

We have now confirmed our reported 95-kDa protein–DHP receptor interaction observed on protein overlays by employing affinity chromatography using a nondenaturing detergent. Furthermore, our chromatography data indicate specifically that the α_1 subunit of the DHP receptor is involved in this interaction; the β subunit does not bind to the 95-kDa protein. Tanabe et al. (1987) predicted from the amino acid sequence that the α_1 subunit of the DHP receptor would have large cytoplasmic domains. Most likely, these cytoplasmic domains are the sites for attachment to the 95-kDa protein, but confirmation requires further study. In order for DHP receptor to bind the 95-kDa protein in vivo, it is necessary (1) that binding occur in the physiological milieu and (2) that binding sites of the two proteins be accessible. Affinity chromatography shows that the DHP receptor is specifically retained by the column in approximately half physiological ionic strength. In addition, we have shown binding under isotonic conditions previously (Kim et al., 1990). The membrane proteins must extend their cytoplasmic domains to make contact over the distance of the junctional gap. Whereas this distance is quite large (120 Å), it is not necessary that the cytoplasmic domains be especially extensive (approximately 80 amino acids could be expected to span the junctional gap as an α helix).

The 95-kDa protein–JFP interaction has been similarly demonstrated (1) in the overlay experiment in which the JFP specifically bound to the 95-kDa protein and (2) in affinity chromatography in which the 95-kDa protein specifically retarded the JFP. Both the 95-kDa protein and JFP are present in the same membrane and approximately colocalize in the junctional region of the TC. Therefore, it is to be expected that the 95-kDa protein and the JFP could also interact in vivo. The strength of interaction of these two proteins is attested to by the observation that the bond is broken only slowly and incompletely in a medium of 1 M NaCl and detergent.

We have demonstrated that both the DHP receptor and JFP specifically bind to a protein of M_r 95 000. In addition to the similar molecular mass, the DHP receptor binding protein and JFP binding protein reveal similar organelle distribution and copurify through three distinct chromatographic steps. Therefore, the DHP receptor binding protein of M_r 95 000 is almost certainly the same protein of M_r 95 000 which binds to the JFP. These findings suggest that this protein may be a candidate for the intermediary protein between the DHP receptor and JFP.

The initial identification of the 95-kDa protein was impeded by the presence of the SR Ca^{2+} pump which has a similar molecular weight. The evidence that the 95-kDa protein is not the Ca^{2+} pump is the lack of binding of the DHP receptor and JFP to a band at $\sim M_r$ 100 000 in the longitudinal reticulum where >90% of the protein is the Ca^{2+} pump. Furthermore, we have separated the 95-kDa protein from the Ca^{2+} pump by HA chromatography. Similarly, the 95-kDa protein is not phosphorylase *b* since the two probes do not bind to a $\sim M_r$ 95 000 band in T-tubules where phosphorylase *b* is present (Dombradi et al., 1984). The electrophorectic mobility of the 95-kDa protein in a number of preparations is slightly variable between M_r 95 000 and 100 000: sometimes the purified 95-kDa protein is coincident with phosphorylase *b* and sometimes above it. In addition, the DHP receptor does not overlay onto phosphorylase *b* in the molecular weight standards.

A protein has recently been described by Zaidi et al. (1989) as a SR Ca^{2+} channel of M_r 106 000. We do not know whether this protein is identical with our M_r 95 000 protein, but the electrophoretic mobilities of the two proteins appear different. We have never observed the DHP receptor or JFP to bind to a band immediately above the Ca^{2+} pump in protein overlays as described by these authors. In addition, the 95-kDa protein does not dissolve well in CHAPS solutions. It may therefore be expected to be present in low concentration when the protocol of Zaidi et al. (1989) is employed for membrane dissolution.

The 95-kDa protein affinity chromatography identified a 170-kDa Stains-all blue staining protein which was specifically retained on the 95-kDa protein column. This protein may be the 170-kDa Stains-all blue protein of the SR first described by Campbell et al. (1983). Several groups have ascribed various functions to the 170-kDa Stains-all blue protein: (1) Zorzato et al. (1986) proposed that the 170-kDa protein is an integral component of the SR Ca^{2+} release channel based on [^{14}C]doxorubicin labeling; (2) Rubtsov and Murphy (1988) suggested that the 170-kDa protein is the caffeine receptor of the SR; (3) Hofmann et al. (1989) reported that a 165-kDa protein showing similar staining and Ca^{2+} binding properties to those of the 170-kDa Stains-all blue protein exhibits characteristics of a plasma lipoprotein receptor. The relationships between our 170-kDa Stains-all blue protein and the 170-kDa proteins cited above remain to be determined. We do not know whether this 170-kDa protein interacts directly with the 95-kDa protein or indirectly through a third protein. Although the role of this 170-kDa protein is not clear, it may play a role in attachment of calsequestrin to the TC membrane. Calsequestrin has been shown to be retained on the JFP affinity column (Kawamoto et al., 1986) and associated with the junctional complex of the TC membrane (Ikemoto et al., 1989).

Figure 8 illustrates our model for the triad junction revised from the previous one (Brandt et al., 1990) as implied by findings in this study. The α_1 subunit of the DHP receptor

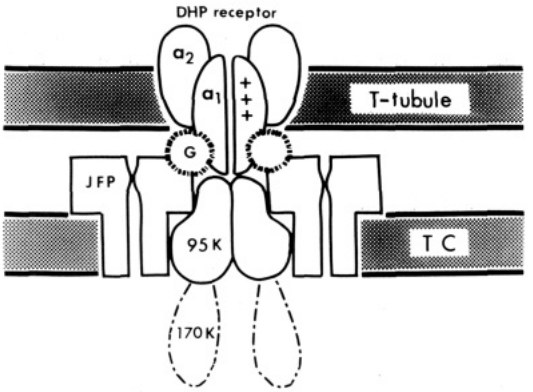

FIGURE 8: Schematic model for the triad junction. The α_1 subunit of DHP receptor is postulated to have voltage-sensing segments [depicted as (+)] in the T-tubular membrane directly interacting with the 95-kDa protein present in the TC, which in turn interacts with the JFP forming the DHP receptor–95-kDa protein–JFP ternary complex. Binding of glyceraldehyde-3-phosphate dehydrogenase (G) to the DHP receptor and JFP is represented as a broken circle. 170K is the Stains-all blue staining protein.

is postulated to have voltage-sensing segments (Tanabe et al., 1987) depicted as (+++). The JFP is postulated to have a large cytoplasmic region and the membrane-spanning region forming the channel (Tanabe et al., 1989; Zorzato et al., 1990). Our previous data indicated that the JFP and the DHP receptor do not interact directly but can associate indirectly through GAPDH or aldolase at the same site. Our new data indicate an interaction through the 95-kDa protein making a second bridging structure.

We propose that primary bridging structure in the junction is the DHP receptor–95-kDa protein–JFP ternary complex. The present study employing anti-DHP receptor subunit-specific antibodies suggests that the α_1 subunit of the DHP receptor is the constituent involved in the DHP receptor–95-kDa protein–JFP ternary complex. The 95-kDa protein is also depicted to interact with the 170-kDa Stains-all blue staining protein which might serve as an anchoring protein of calsequestrin. A secondary bridging structure is the DHP receptor–GAPDH–JFP complex which has been demonstrated previously (Brandt et al., 1990). GAPDH has been shown to promote junction formation of the isolated T-tubule with the terminal cisternae (Corbett et al., 1985) and to bind to both the JFP and the DHP receptor primarily by ionic bonds (Brandt et al., 1990). Therefore, in the normal intracellular millieu, the strength of DHP receptor–GAPDH–JFP association is likely to be weak while the predominant bridging structure, the DHP receptor–95-kDa protein–JFP complex, can be maintained in hypertonic solution. Existence of the two potential bridges in the triad junction may be correlated to the two populations of isolated triads which differ in their susceptibility to the breakage procedures (Kim et al., 1990). It is possible that the DHP receptor–95-kDa protein–JFP complex forms the structural basis for the strong triads and the DHP receptor–GAPDH–JFP forms the weak triads.

The model presented here is purely an architectural model of the triad junction. The putative extensive cytoplasmic domains of the α_1 subunit of the DHP receptor may form a filamentous structure joining to the 95-kDa protein which may be related morphologically to the previous reported junctional bridges (Somlyo, 1979), pillars (Eisenberg & Gilai, 1979), or thin filamentous connections observed in the strong triad junction (Kim et al., 1990). The continuation of the DHP receptor–95-kDa protein complex to the 170-kDa blue-staining protein might be correlated with the rodlike structure seen in freeze–fracture image of the TC (Dulhunty, 1987). The 170-kDa blue-staining protein might be a constituent of the thin strands connecting the calsequestrin to the junctional SR observed in a deep-etch study (Franzini-Armstrong et al., 1987). The central position of the 95-kDa protein between two recognized components of EC coupling suggests that this protein may also play an essential physiological role.

ACKNOWLEDGMENTS

We thank S.-R. Wen and H. Doobay for preparation of the DHP receptor antibodies.

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Equilibrium Denaturation of Insulin and Proinsulin

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Received March 15, 1990; Revised Manuscript Received June 27, 1990

ABSTRACT: The guanidine hydrochloride induced equilibrium denaturation of insulin and proinsulin was studied by using near- and far-ultraviolet (UV) circular dichroism (CD). The denaturation transition of insulin is reversible, cooperative, symmetrical, and the same whether detected by near- or far-UV CD. These results are consistent with a two-state denaturation process without any appreciable equilibrium intermediates. Analysis of the insulin denaturation data yields a Gibbs free energy of unfolding of 4.5 ± 0.5 kcal/mol. Denaturation of proinsulin detected by near-UV CD appears to be the same as for insulin, but if detected by far-UV CD appears different. The far-UV CD results demonstrate a multiphasic transition with the connecting peptide portion unfolding at lower concentrations of denaturant. Similar studies with the isolated C-peptide show that its conformation and susceptibility to denaturation are independent of the rest of the proinsulin molecule. After the proinsulin denaturation results were adjusted for the connecting peptide contribution, a denaturation transition identical with that of insulin was obtained. These results show that for proinsulin, the connecting peptide segment is not a random coil; it is an autonomous folding unit, and the portion corresponding to insulin is identical with insulin in terms of conformational stability.

Insulin is a 2-chain (A and B) globular protein of 51 amino acids containing 1 intrachain and 2 interchain disulfide bonds (Brown et al., 1955). Extensive X-ray crystallographic studies of insulin have been carried out and have provided detailed information concerning its secondary, tertiary, and quaternary structures (Adam et al., 1969; Baker et al., 1988; Chang et al., 1986; Wang et al., 1982). Insulin's secondary structure is approximately 40% helical with helices spanning residues A1–A5 and A13–A19 of the A chain and B9–B19 of the B chain. An extended chain persists from B1–B8 and B21–B30 of the B chain. Several crystal forms have been studied by X-ray diffraction (Bentley et al., 1976; Dodson et al., 1978). Each crystal structure differs slightly, with the principle difference lying at the N- and the C-terminus of the B chain.

The solution behavior of insulin is known to be complex. The metal-free species exhibits a pH- and concentration-dependent association pattern consisting of monomer, dimer, tetramer, etc., all in dynamic equilibrium (Fredericq, 1956; Jeffrey & Coates, 1966; Pekar & Frank, 1972; Goldman & Carpenter, 1974; Jeffrey et al., 1976; Pocker & Biswas, 1981). Zn and other divalent metal ions induce the specific formation of an insulin hexamer (Fredericq, 1956; Cunningham et al., 1955). The association and crystallization behavior of insulin has been utilized by the pharmaceutical industry to effect changes in the time action of insulin formulations (Davidson, 1980). Highly aggregated or crystalline formulations are slower to dissociate and result in extended action. The active form is thought to be monomeric (Frank et al., 1972a). Insulin association has been studied by a wide range of spectral and physical techniques such as light scattering (Bohidar & Geissler, 1984), optical spectroscopy (Rupley et al., 1967; Pocker & Biswas, 1980; Goldman & Carpenter, 1974), ultracentrifugation (Jeffrey & Coates, 1966; Pekar & Frank, 1972; Goldman & Carpenter, 1974; Mark et al., 1987), and equilibrium dialysis (Grant et al., 1972; Goldman & Carpenter 1974). There are no dramatic changes in the insulin conformation upon dilution to the monomeric state, but small changes, of unknown significance, have been observed (Pocker & Biswas, 1980; Goldman & Carpenter, 1974).

Insulin is derived from a single-chain precursor, proinsulin (Steiner & Oyer, 1967). A 35 amino acid fragment designated as the connecting peptide is removed from the middle of proinsulin to form insulin (Steiner et al., 1971). The biological activity of proinsulin is only about 10–20% that of insulin (Peavy et al., 1985). The conformation of proinsulin has not been determined by X-ray diffraction, but optical spectroscopy studies support the contention that the insulin portion of the proinsulin molecule is in the same or very similar conformation

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0006-2960/90/0429-9289$02.50/0