Ca^{2+}-Calmodulin Binding to Mouse α1 Syntrophin: Syntrophin Is Also a Ca^{2+}-Binding Protein†

Bobby J. Newbell, J. Todd Anderson, and Harry W. Jarrett*

Department of Biochemistry, 858 Madison Avenue, University of Tennessee, Memphis, Tennessee 38163

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ABSTRACT: Syntrophins are peripheral membrane proteins which have been found associated with dystrophin, the protein product of the Duchenne muscular dystrophy gene locus. Mouse α1 syntrophin binds the COOH-terminal domain of dystrophin, and calmodulin inhibits this interaction in a Ca^{2+}-dependent fashion. Where calmodulin binds to syntrophin was investigated by constructing fusion proteins containing different regions of syntrophin's sequence. Syntrophin contains at least two regions which bind calmodulin in different ways. The COOH-terminal 24 residues contain a Ca^{2+}-calmodulin binding site, named CBS-C, which binds calmodulin with an apparent affinity of 18 nM and which is highly conserved in all syntrophins. The amino-terminal 174 residue section of syntrophin contains other calmodulin binding, and binding occurs in either the presence or absence of Ca^{2+} with an apparent affinity of 100 nM. Syntrophin was shown to bind Ca^{2+} at two or more sites residing in the amino-terminal 274 residues, and Ca^{2+} binding to syntrophin affects calmodulin binding at high concentrations of syntrophin. Syntrophin A (residues 4−274) is predominantly a dimer in EGTA. A model of syntrophin's complex interactions with itself (i.e., oligomerization), calmodulin, and Ca^{2+} is presented.

Syntrophins are peripheral membrane proteins first identified in the postsynaptic membrane of *Torpedo* (Froehner, 1984). Subsequently, syntrophins were found to be closely associated with dystrophin, the protein product of the Duchenne muscular dystrophy gene locus (Kramarcy et al., 1994). In skeletal muscle, dystrophin and syntrophins are found in a complex with other proteins and glycoproteins, the dystrophin glycoprotein complex or DGC[1] (Yoshida & Ozawa, 1990; Ervasti & Campbell, 1991). The syntrophins are homologous proteins of which three are known, the α1, *β*1, and *β*2 syntrophins. The α1 syntrophin is expressed primarily in striated muscle and brain, while the *β*-syntrophins are ubiquitous in mammalian tissues (Ahn, et al., 1996). The domain structure of the syntrophins (Gibson et al., 1994; Adams et al., 1995) contains two pleckstrin homology (PH) domains, a PDZ domain homologous to the postsynaptic density 95 kDa protein, and a unique syntrophin domain (SU). The PDZ domain is inserted into and interrupts the first PH domain. This domain structure is suggestive of membrane association and of a role of signal transduction in modulating syntrophin's function.

Signal transduction is likely to operate at multiple locations in the DGC. Calmodulin binds dystrophin, syntrophins, and α-dystroglycan, all members of this complex (Madhavan et al., 1992). Dystrophin binds F-actin (Way et al., 1992; Fabrizzio et al., 1993; Corrado et al., 1994; Jarrett & Foster, 1995), and calmodulin inhibits this interaction (Jarrett & Foster, 1995) though not under some assay conditions (Bonet-Kerrache, et al., 1994). Syntrophins bind dystrophin protein sequences derived from dystrophin's exon 73 and 74 sequences (Ahn & Kunkel, 1995; Yang et al., 1995a; Suzuki et al., 1995). Nearby, calmodulin binds sequences derived from dystrophin's exon 69 with high affinity, suggesting that calmodulin may potentially regulate the dystrophin−syntrophin interaction (Anderson, et al., 1996). Syntrophin also binds DGC α- and *γ*-sarcoglycan, though much less is known about this binding (Madhavan & Jarrett, 1995a). α-Syntrophin also self-associates and associates with other syntrophins (Madhavan & Jarrett, 1995a) though this oligomerization is only poorly characterized. Both dystrophin and syntrophin are phosphorylated *in vivo* (Milner et al., 1993; Wagner & Huganir, 1994) and *in vitro* by CaM kinase II (Madhavan & Jarrett, 1994) and, for dystrophin, by cdc2 kinase (Milner et al., 1993); sites of phosphorylation have been localized to regions near where dystrophin and syntrophin interact (Madhavan & Jarrett, 1995b; Walsh et al., 1995). Two other signaling proteins also interact with the DGC. Nitric oxide synthase associates with the DGC (Brenman et al., 1995), by direct interaction with syntrophin (Brenman, et al., 1996) and DGC *β*-dystroglycan has been shown to bind the grb2 adapter protein (Yang et al., 1995b).

While calmodulin binding to DGC syntrophins on gel blots has been demonstrated (Madhavan & Jarrett, 1992), the location of this binding to specific syntrophin sequences and the functional significance of binding remain to be elucidated. Syntrophin is present in only limited amounts from muscle and other tissues, and purification would require detergents and harsh, alkaline conditions (Ervasti & Campbell, 1991). Because of these limitations, we have used bacterial expression of chimeric fusion proteins to investigate syntrophin. Here, we show that syntrophin binds calmodulin in both the presence and absence of calcium and these binding events occur at separate sites. Calcium-dependent binding occurs

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* To whom correspondence should be addressed. FAX: (901) 448-7360; Phone: (901) 448-7078; e-mail: hjarrett@utmem1.utmem.edu.

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[1] Abbreviations: DGC, dystrophin glycoprotein complex; MBP, maltose-binding protein; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

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in the COOH-terminal 24 amino acid residues of syntrophin in the SU domain. Calcium-independent binding was not completely localized but resides in the amino-terminal 173 residues of syntrophin. Furthermore, calmodulin antagonizes the dystrophin−syntrophin interaction *in vitro*. Additionally, we show for the first time that syntrophin binds Ca^{2+}.

EXPERIMENTAL PROCEDURES

Fusion Proteins. The unique COOH-terminal domain of dystrophin, DysS9, was expressed as a chimeric fusion with maltose-binding protein (MBP) as previously described (Madhavan & Jarrett, 1995a). **Syn**: Syntrophin was expressed as a chimeric fusion protein from the pMalc plasmid as an MBP fusion or from pET28 as a His-Tag fusion protein. Construction of both plasmids and transformation of bacteria have been previously described (Madhavan & Jarrett, 1995a). In either case, the fusion protein contains mouse α1 syntrophin 4−503 protein sequences. **Syn A**: Syn in pMalc was restricted at the unique insert *Alw*NI site, and the overhang was blunted with the Klenow large fragment DNA polymerase I 3′-exonuclease activity. After addition of EDTA to 10 mM, heating to 75 °C for 10 min, phenol/chloroform extraction, and ethanol precipitation, the blunted DNA was restricted with *Bam*HI at the unique upstream plasmid site, gel purified, and ligated into *Bam*HI/*Stu*I pMalf restricted vector, which provided the necessary stop codon. The pMalf plasmid has been previously described (Jarrett & Foster, 1995). Syn A was also subcloned into pET28a. Syn A in pMalf was restricted at the plasmid *Bam*HI upstream and *Hin*dIII downstream sites. The resulting 806 bp fragment was gel purified and ligated into a *Bam*HI/*Hin*dIII pET 28a(+) vector. **Syn B**: A portion of the *Alw*NI restricted Syn prepared for Syn A was restricted at the downstream plasmid *Xba*I site, and the resulting 969 bp piece was gel purified and ligated into a *Stu*I/*Xba*I pMalf vector. **Syn C**: Syn in pMalc was restricted at the unique insert *Eag*I site, and the overhang was filled using Klenow. After denaturing the Klenow and ethanol precipitating, the DNA was restricted with *Bam*HI at the upstream unique plasmid site, gel purified, and ligated into *Bam*HI/*Stu*I pMalf restricted vector which provided the necessary stop codon. **Syn D**: A portion of the filled, *Eag*I restricted Syn prepared for Syn C was restricted at the downstream plasmid *Xba*I site, and the resulting 545 bp piece was gel purified and ligated into a *Stu*I/*Xba*I pMalc vector. **Syn E**: Syn in pMalc was restricted at the unique insert *Bsp*MI site, and the resulting overhang was filled using Klenow. After denaturing the Klenow and ethanol precipitating, the DNA was restricted with *Bam*HI at the upstream unique plasmid site. This yielded a 1436 bp fragment that was gel purified and ligated into *Bam*HI/*Stu*I pMalf restricted vector which provided the stop codon. **Syn F**: A portion of the filled, *Bsp*MI restricted Syn prepared for Syn E was restricted at the downstream plasmid *Xba*I site, and the resulting 563 bp piece was gel purified and ligated into a filled *Eco*RI/*Xba*I pMalc vector. **Syn G**: Syn E in pMalf was restricted at the unique insert *Alw*NI site, and the overhang was blunted with the 3′-exonuclease activity of Klenow. After denaturing the Klenow and ethanol precipitating, the DNA was restricted at the downstream plasmid *Xba*I site, and the resulting 615 bp piece was gel purified and ligated into a *Stu*I/*Xba*I pMalc vector. **Syn H**: Syn A in pMalf was restricted with *Eco*RI which cuts in the insert and downstream in the plasmid yielding a 317 bp piece. The resulting overhangs were filled using Klenow. After denaturing the Klenow and ethanol precipitating, the plasmid DNA was restricted with *Bam*HI at the unique upstream plasmid site, and the resulting 502 bp fragment was gel purified and ligated into *Bam*HI/*Stu*I pMalf restricted vector which provided the stop codon. **Syn I**: The 317 bp filled *Eco*RI fragment produced from Syn H was gel purified and ligated into a *Stu*I/filled *Eco*RI pMalc vector which provided the correct reading frame for a stop codon. Each protein was expressed in *Escherichia coli* and affinity purified as previously described (Jarrett & Foster, 1995; Madhavan & Jarrett, 1995a). The syntrophin fusion proteins were stored at −20 °C in 15% glycerol.

Equilibrium Dialysis. His-Tag-syntrophin A (0.55 mg/mL) and His-Tag-syntrophin B (0.5 mg/mL) as well as several other fusion proteins were subjected to equilibrium dialysis to measure Ca^{2+} binding. Each protein (0.2 mL) was dialyzed versus two changes of 100 mL of 10 mM MOPS, pH 7, 90 mM KCl, and 50 μM $CaCl_2$ containing 10 μCi of ^{45}Ca. After dialysis, 10 μL of each protein sample and the dialysis solution were subjected to scintillation counting (5 mL of scintillation cocktail), and all measurements were of triplicate samples. The protein concentration of each sample after dialysis was determined by the method of Bradford (1976) with bovine serum albumin as the standard. At least two separate experiments were performed for each protein.

Calmodulins. Calmodulin was purified and calmodulin−Sepharose, dansyl-calmodulin, and biotinylated-calmodulin were prepared as previously described (Anderson, et al., 1996). The dansyl-calmodulin contains 1.1 mol of dansyl moiety/mole calmodulin. Emission spectra of dansyl-calmodulin (25 nM) and measurements of dansyl-calmodulin (9 nM) affinity were also as previously described (Anderson et al., 1996) either in buffer MC (10 mM MOPS, pH 7, 90 mM KCl, 1 mM $CaCl_2$) or in buffer ME (the same except 1 mM EGTA replaces the $CaCl_2$). Both buffers additionally contained 0.1 mg/mL ovalbumin.

Solid Phase Immunoassay. To measure the binding of His-Tag-syntrophin to MBP-DysS9, a microtiter assay was used. The microtiter plate (Dynatech Immulon 1) was coated by incubation of 100 μL/well of 0.05 mg/mL His-Tag-syntrophin in buffer AC8 (50 mM Tris-HCl, pH 7.5, 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM $CaCl_2$) at 4° for 4 h. The plate was then blocked by replacing the His-Tag-syntrophin solution with 300 μL/well of 30 mg/mL bovine serum albumin in AC8T at 4° overnight. All subsequent operations were at room temperature (20°) and 100 μL/well. The plate was then washed three times (5 min per wash) with buffer AC8T-BSA (AC8 containing 0.1% Tween-20 and 1 mg/mL bovine serum albumin). After removing the last wash, serial dilutions of MBP-DysS9 were added. In some experiments calmodulin was included in the MBP-DysS9 dilutions at a fixed concentration shown in the figures. The plate was incubated for 2 h. The plate was then washed three times with AC8T-BSA as described above and then incubated for 1 h with 1:1000 anti-MBP rabbit serum diluted with AC8T-BSA. The plate was then washed three more times as described and then incubated with 1:500 goat anti-rabbit alkaline phosphatase conjugates (Bio-Rad Laboratories) prepared in AC8T-BSA for 1 h. Subsequent washes were for 10 min. The wells were then washed once with AC8T-BSA and twice with 10 mM diethanolamine/0.5 mM $MgCl_2$.

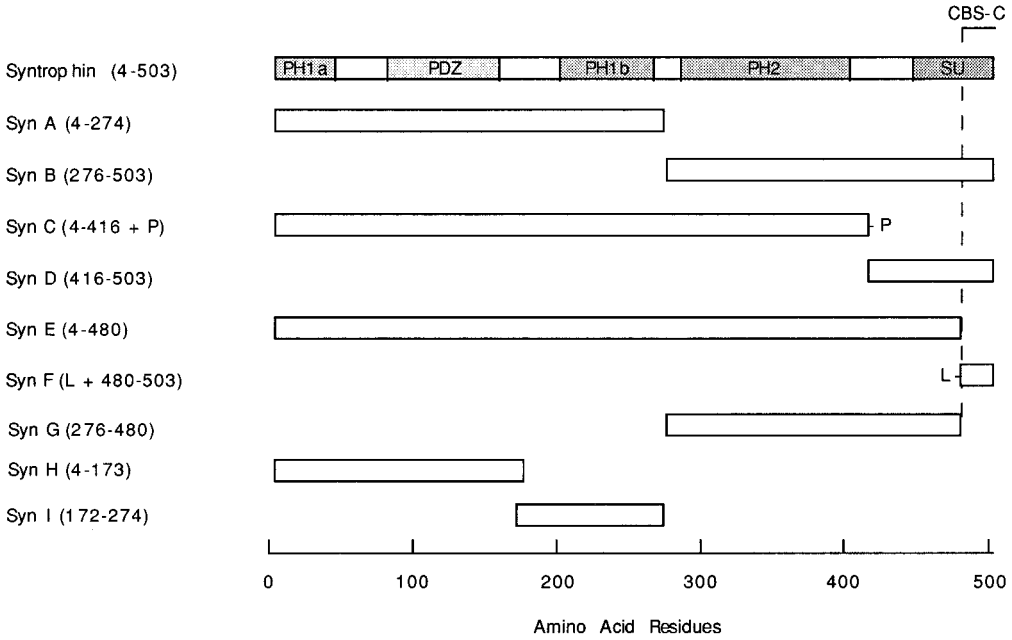

FIGURE 1: Fusion proteins containing α1 syntrophin sequences. Syntrophin sequences produced as fusion proteins are shown to scale along with the domain structure of syntrophin. Numbers in parentheses give the syntrophin amino acid sequences present in each fusion as well as any extra (non-syntrophin) amino acids present at either terminus. CBS-C indicates a putative calmodulin-binding sequence.

To each well was added 100 μL of 1 mg/mL *p*-nitrophenyl phosphate in 10 mM diethanolamine/0.5 mM $MgCl_2$, and absorption at 405 nm was recorded every 5 min until 30 min had elapsed. The change in absorption over a 10 min period was calculated from the initial linear rate of change. As a control, each experiment was also repeated with plates which were not coated with His-Tag-syntrophin, and these controls showed no significant binding.

This experiment was also repeated with 1 mM EGTA replacing $CaCl_2$ in all of the buffers up to the stage at which the anti-MBP antibody was added. After that stage, the experiment was continued as specified above using the $CaCl_2$ containing AC8T-BSA.

Size exclusion chromatography was performed using a 0.75 × 30 cm Beckman SEC 3000 column equilibrated in buffer ME. His-Tag-syntrophin A (16.9 μM) was dialyzed into buffer ME, and 50 μL was injected onto the column. Ovalbumin and bovine serum albumin were used as molecular mass standards.

Mathematical Apparatus. Binding or fluorescence data were fit by nonlinear least squares analysis (using the PSI-Plot software and the Marquardt algorithm) to various mathematical models. These include modified Adair−Scatchard equations (Adair, 1925; Klotz, 1946) for one, two, and three binding sites (for Figure 5 and Table 1) and a model for simple, competitive inhibition (Figure 3) to describe calmodulin inhibition of the syntrophin−dystrophin interaction:

$$\frac{A_{405}}{A_{405,Max}} = \frac{[DysS9]}{K_{Dys}(1 + [CaM]/K_I) + [DysS9]}$$

where A_{405} is the absorption at 405 nm obtained for each concentration of dystrophin fusion protein, [DysS9], and $A_{405,Max}$ is the maximal absorption. K_{Dys} and K_I are the apparent dissociation constants for DysS9 binding to syntrophin and calmodulin (CaM) binding as an inhibitor, respectively.

RESULTS

Syntrophin Fusion Proteins. Syntrophin sequences expressed as fusion proteins are depicted in Figure 1. Many of these were produced as both His-Tag and maltose-binding protein fusions (MBP) because some did not express well in one system or the other. Several controls including the Mal F protein (which contains only MBP sequences), a His-Tag-β-galactosidase, and His-Tag fusion containing sequences from collagenase were also expressed and were shown to be negative for activities discussed in this paper. Furthermore, whenever possible, results obtained with each fusion protein were confirmed in the alternate system; i.e., MBP and His-Tag fusions of the same syntrophin sequences were used in replicate experiments.

Each fusion protein was expressed and affinity purified. Figure 2 shows that the purified protein in most cases was full-length, but several also showed some smaller protein species which result from partial proteolysis. This was demonstrated by showing that every protein species present on gel blots contained either the MBP or His-Tag sequences detected with the appropriate, specific antibodies (data not shown). This partial proteolysis had also been encountered with dystrophin fusion proteins (Jarrett & Foster, 1995; Anderson et al., 1996). Two of the fusion proteins (syntrophin C and I) are not shown in Figure 2. Both were also of the expected size and were typical of the other fusion proteins shown except that syntrophin C showed somewhat greater proteolysis (data not shown).

Ca^{2+}-Calmodulin Inhibits the Dystrophin−Syntrophin Interaction. Syntrophin binds dystrophin (Kramarcy et al., 1994); binding occurs in the COOH-terminal domain of dystrophin (Ahn & Kunkel, 1995; Yang et al., 1995a; Suzuki et al., 1995). This domain of dystrophin has been expressed as an MBP fusion protein called DysS9 and shown to bind to skeletal muscle syntrophin on gel blots of purified dystrophin glycoprotein complex (Madhavan & Jarrett, 1995a). Conversely, the His-Tag-syntrophin fusion protein used here has been shown to bind DGC dystrophin (Madha-

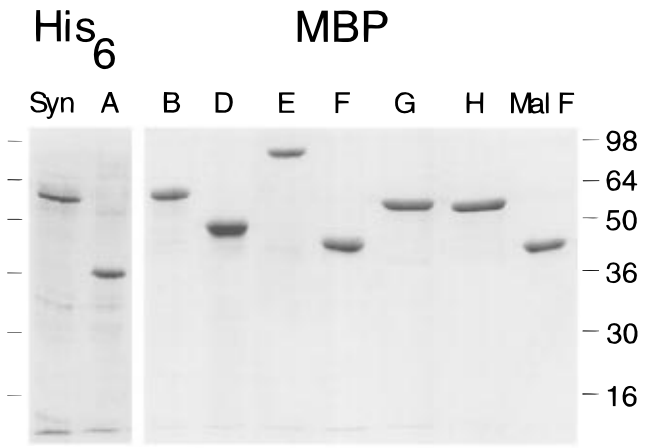

FIGURE 2: Each of the purified fusion proteins is of the expected size. One microgram of each fusion protein shown was applied to 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue. The position of molecular weight markers is given along with their M_r in kilodaltons. The left side shows His-Tag fusion proteins produced using the pET28 expression system, while the right side shows some of the maltose-binding protein (MBP) fusion proteins produced using pMal.

van & Jarrett, 1995a). To determine if calmodulin affects this interaction, His-Tag-syntrophin fusion protein was used to coat the wells of a microtiter plate, and the binding of MBP-DysS9 was assessed in the presence and absence of calmodulin. For this experiment, all solutions contained 1 mM $CaCl_2$. Binding was detected using the anti-MBP antibody. A typical experiment is presented in Figure 3. Averaging data from three separate experiments gave half-maximal binding at 4.0 ± 0.3 μg/mL DysS9 (47 ± 4 nM). Calmodulin inhibits the syntrophin−DysS9 interaction, and these data were fit by nonlinear least squares analysis to the equation for simple competitive inhibition. The fit was reasonably good, as indicated by the correlation coefficient (r) of 0.98. This fit to the competitive inhibition model is also indicated by the double reciprocal plot (see inset, Figure 3) showing that calmodulin affects the apparent dissociation constant without affecting the maximal amount of binding. The inhibition constant (K_I) derived from three separate experiments is 343 ± 35 nM calmodulin.

This experiment was also repeated with EGTA replacing Ca^{2+} during the binding of DysS9 to syntrophin. The result was that DysS9 still bound syntrophin with approximately the same affinity, but calmodulin no longer inhibited binding (data not shown). Thus, calmodulin inhibition requires the presence of Ca^{2+} while the syntrophin−dystrophin interaction does not require Ca^{2+}. Presumably, Ca^{2+}-calmodulin is required for inhibition.

Where calmodulin binds to inhibit this interaction is not yet clear. Ca^{2+}-calmodulin binds to the DysS9 sequences of dystrophin. The affinity of this interaction has been measured with dansyl-calmodulin, and DysS9 binds with an apparent affinity of 54 nM (Anderson et al., 1996). Since this affinity does not closely match the apparent inhibition constant (343 nM), this suggests that Ca^{2+}-calmodulin does not inhibit by simple binding to dystrophin. Calmodulin has also been shown to bind to DGC syntrophins. The affinity of this interaction has been reported to be in the range of 6−60 nM (Madhavan et al., 1992). We next characterized the binding of syntrophin fusion proteins to calmodulin.

Localizing α-Syntrophin's Calmodulin-Binding Sites. Figure 4 shows the results obtained when syntrophin fusion

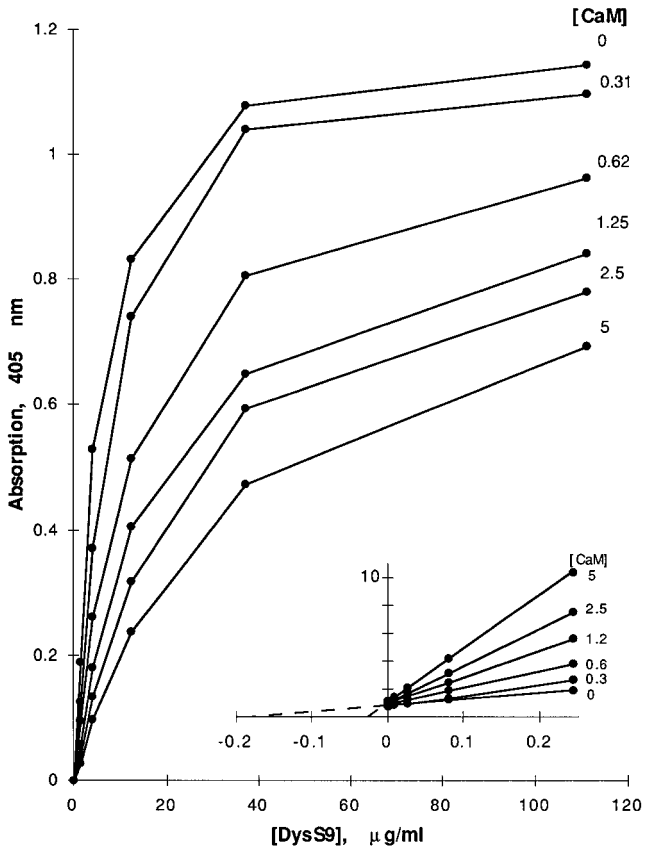

FIGURE 3: Calmodulin inhibits the syntrophin−dystrophin interaction. Absorption 405 nm measures the amount of MBP−DysS9 dystrophin fusion protein which bound to a His-Tag-syntrophin coated microtiter plate. Calmodulin concentrations (micromolar) added along with the MBP−DysS9 fusion are shown in the figure. The inset shows a double reciprocal plot of these data.

proteins were applied to calmodulin−Sepharose in the presence of Ca^{2+} and eluted with EGTA. The results in Figure 4 demonstrate that calmodulin binding occurs at both amino- (e.g., Syn A) and COOH-terminal (e.g., Syn B) regions of syntrophin's sequence. The COOH-terminal binding apparently resides within the COOH-terminal 24 residues of syntrophin in the syntrophin unique domain (see Figure 1, CBS-C). This COOH-terminal calmodulin-binding site (CBS-C) probably accounts for all of the calmodulin binding in syntrophin B since all fusions containing this sequence (syntrophin B, D, F) bind to calmodulin−Sepharose, while syntrophin G, which contains all of the syntrophin B sequences except CBS-C, does not bind. The amino-terminal binding was not as well characterized but apparently resides in syntrophin 4−173 since all fusions containing these sequences also bind to calmodulin−Sepharose. The number of binding sites in the amino-terminus was not determined.

For those proteins which do bind calmodulin−Sepharose, some fraction of each protein is not bound by the column and passes through unretained (Figure 4). This was investigated further. The amount retained was variable when different preparations of the same protein were analyzed—some syntrophin samples were nearly completely retained by the calmodulin−Sepharose column while others showed less binding. When the unretained and eluted proteins were examined by sodium dodecyl sulfate−polyacrylamide electrophoresis, the two samples did not appear different in the relative amounts of intact and proteolyzed protein. When

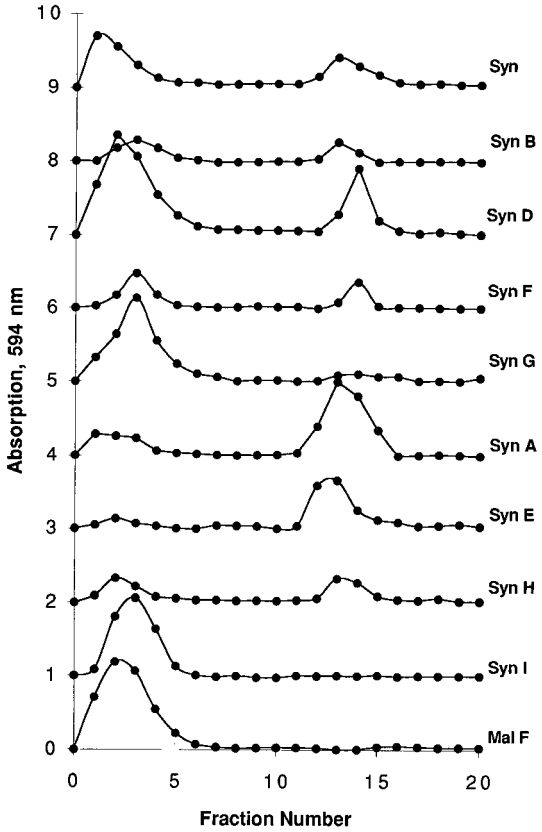

FIGURE 4: Fusion proteins containing syntrophin sequences 4−173 and 480−503 bind to calmodulin−Sepharose in a Ca^{2+}-dependent manner. Chromatography of 0.2 mg of each fusion protein on a 2 mL calmodulin−Sepharose column is shown. Ca^{2+} was present in all buffers initially and up to fraction number 11 when it was replaced with EGTA to elute the column. Protein was detected using the Bradford dye binding assay (Bradford, 1976) by absorption at 594 nm. The chromatograms from different experiments are displaced by 1 absorption unit so that they may be shown on the same figure for comparison. For this experiment, syntrophin (Syn), Syn A, Syn B, and Syn I were His-Tag fusion proteins; all others were MBP fusion proteins.

twice as much syntrophin was applied to the column, twice as much was bound and eluted, showing that the calmodulin−Sepharose had adequate capacity to bind all of the applied syntrophin. When the syntrophin eluted from the column with EGTA was mixed with excess calcium and reapplied to the column, all of it bound and eluted in EGTA (data not shown). We conclude that purified syntrophin fusion protein contains a variable fraction which does not bind calmodulin and is probably improperly folded. That fraction which does bind to calmodulin can be recovered from chromatography and all binds upon rechromatography, indicating that properly folded syntrophin remains properly folded (data not shown). This same behavior was also observed with dystrophin COOH-terminal sequences (Anderson et al., 1996); again, a fraction was improperly folded while the remainder bound calmodulin.

Calmodulin-Binding Affinity of Syntrophin Sequences. Given the potential for improper folding and evidence for partial proteolysis already discussed, it would be difficult to accurately determine the calmodulin-binding affinities of syntrophin; however, here we present a more qualitative, comparative approach to learn where high affinity binding resides. The concentration required to obtain a 50% increase in the dansyl-calmodulin fluorescence, $K_{0.5}$, was determined (in the presence of calcium) as shown in Figure 5. When

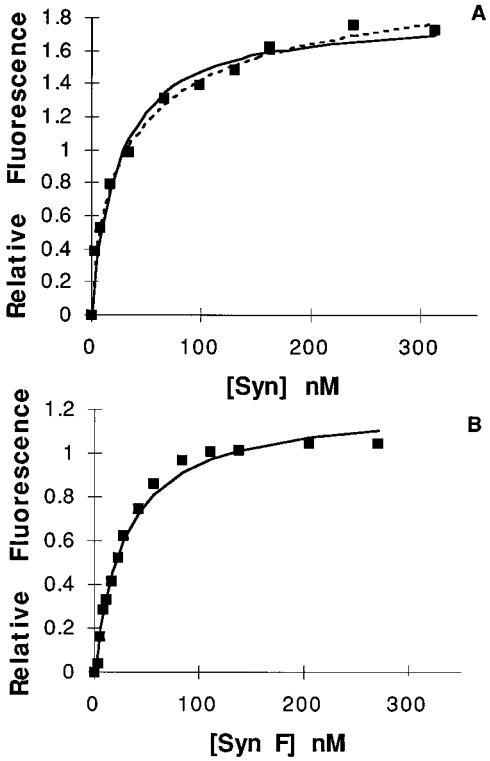

FIGURE 5: Syntrophin and syntrophin F have similar high affinity dansyl-calmodulin binding. Nine nanomolar dansyl-calmodulin in buffer MC containing 0.1 mg/mL ovalbumin was titrated with additions of the syntrophin fusion proteins and fluorescence measured (closed squares, 345 nm excitation, 490 nm emission). The data from an identical titration in the absence of dansyl-calmodulin have been subtracted to correct for fusion protein fluorescence, and the fluorescence in the absence of fusion protein was subtracted to show only the fluorescent changes resulting from syntrophin sequences. "Relative Fluorescence" is the photomultiplier tube output in volts on a scale where the fluorescence of dansyl-calmodulin was set at 6 V; e.g., a relative fluorescence of 1.5 V represents a 25% increase in the fluorescence. The lines shown are those obtained from fitting these data by nonlinear least squares analysis (using the PsiPlot software) to either a single-site Scatchard−Adair (solid line) or to a two-site Adair equation (dashed line). Shown are the data for His-Tag-syntrophin (panel A) and MBP-syntrophin F (panel B).

dansyl-calmodulin is excited at 345 nm, the emission at 490 nm is affected by interaction with calmodulin-binding proteins (Kincaid et al., 1982; Johnson & Wittenauer, 1983) and may be used to measure the apparent affinity of this interaction (Anderson et al., 1996). Fluorescent binding data for syntrophin and syntrophin F are presented in Figure 5. The solid line shown is that obtained by fitting the data by nonlinear least squares analysis to the single-site Scatchard−Adair equation. The apparent dissociation constants derived from fitting these data to this simple model are similar for syntrophin (Figure 5A, 23 nM) and syntrophin F (Figure 5B, 28 nM). Thus, high affinity binding resides in the CBS-C sequences contained in syntrophin F. This was also confirmed when more complex binding models were used (see below). When similar experiments were performed with the Mal F and syntrophin G fusion proteins, the fluorescence was not significantly affected (data not shown), demonstrating that the fluorescence change shown is due to the calmodulin-binding sequences of syntrophin. This result also agrees with the calmodulin−Sepharose experiment (Figure 4), which showed that neither of these proteins bind calmodulin.

Table 1: Apparent Affinity of the Syntrophin Fusion Proteins for Dansyl-calmodulin

protein	no. of determinations	$K_{0.5}$, nM (±SD)
syntrophin[a]	3	18 ± 6
syntrophin A	3	98 ± 9
syntrophin B	3	18 ± 3
syntrophin D	2	42 ± 4
syntrophin E	1	82
syntrophin F	3	32 ± 6

[a] $K_{0.5}$ was determined using the same single-site model for all proteins shown and the values from the different experiments averaged. Syntrophin's data were also fit to other models as described in the text. For these experiments, syntrophin and syntrophin A were His-Tag, all others were MBP fusions.

Experiments such as that in Figure 5 were repeated several times for each syntrophin fusion protein and the data fit to the simple one-site model; the results are presented in Table 1. Because of concerns about proper folding and partial proteolysis discussed above, as well as concerns about the adequacy of a single-site model in all cases (see below), these should be regarded as only estimates of the true dissociation constants. The values reveal, however, that the highest affinity binding ($K_{0.5}$ = ~20 nM) resides in CBS-C (contained in syntrophins, B, D, and F) while lower affinity ($K_{0.5}$ = ~100 nM) binding resides in amino-terminal sequences (syntrophins A and E).

Since syntrophin must contain at least two calmodulin-binding sites to account for the data in Figure 4 and Table 1, the binding data for syntrophin were fit by nonlinear least squares analysis to one-, two-, and three-site binding models based upon the Adair equation. The fit to the one-site (solid line; correlation coefficient, $r = 0.993$) and two-site (dotted line; $r = 0.997$) are illustrated in Figure 5A. The two-site model is the simplest model consistent with all the data and yields (Figure 5A) apparent affinities of 17 and 69 nM. These values found with full-length syntrophin correspond reasonably well with the ~20 and ~100 nM affinities found by expressing only regions of syntrophin's sequence (Table 1). Thus, the data show a high degree of internal consistency. However, both of the more complex (two- and three-site) models fit the data slightly better than the one-site model, and the difference is not statistically significant in either case. More complex equations frequently do fit data better because they allow greater degrees of freedom to the fitting process. Thus, while syntrophin must have at least two calmodulin-binding sites to account for the data presented in Figure 4 and Table 1, the actual number of sites is not yet clear and could not be obtained from curve fitting/model testing.

Table 2 presents a comparison of the calmodulin-binding sequence localized to syntrophin's COOH-terminus and the three binding sites identified in dystrophin (Jarrett & Foster, 1995; Anderson et al., 1996). Calmodulin-binding sequences found in different proteins are not strictly homologous to one another but do all have the characteristic of being cationic, amphipathic sequences which are typically about 20 residues long (Jarrett & Madhavan, 1991). A particularly well studied example of this motif is the calmodulin-binding site of skeletal muscle myosin light chain kinase (see Table 2). The complex of this peptide bound to calmodulin has been characterized by multidimensional NMR and shows that both the positive charges and hydrophobic amino acid side chains interact directly with calmodulin (Ikura et al., 1992; Meador et al., 1992). Syntrophin CBS-C is also cationic and amphipathic. Furthermore, 96% of the CBS-C residues show only conservative replacements, and 61% are identical in all known syntrophins. Dystrophin and utrophin are homologous proteins, and both bind calmodulin (Jarrett & Foster, 1995; Winder & Kendrick-Jones, 1995; Anderson et al., 1996). Three calmodulin-binding sequences have been localized in mouse dystrophin to specific sequence locations (Jarrett & Foster, 1995; Anderson et al.,1996), and all three also show this cationic, amphipathic motif which is also well conserved in utrophin and across species (Table 2).

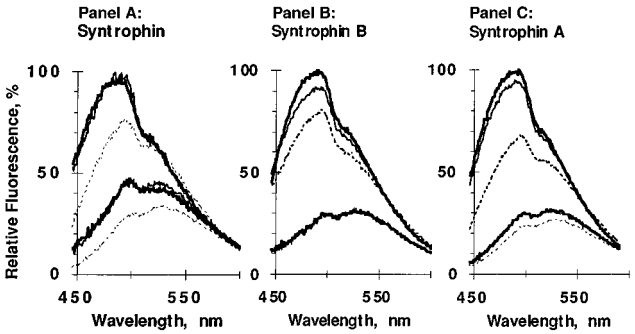

FIGURE 6: Syntrophin fusion proteins cause characteristic changes in the emission spectrum of dansyl-calmodulin. In each panel, the three lower tracings were all obtained in 1 mM EGTA (buffer ME); the upper three tracings are in 1 mM $CaCl_2$ (buffer MC). All buffers additionally contained 0.1 mg/mL ovalbumin. The spectra shown are of 25 nM dansyl-calmodulin containing either no addition (dotted line), 200 nM (thin line), or 400 nM (thickest line) His-Tag-syntrophin (panel A), His-Tag-syntrophin B (panel B), or His-Tag-syntrophin A (panel C) fusion protein. Excitation was at 345 nm, and both excitation and emission slits were set at 8 nm bandpass. The methods used have been previously described (Anderson et al., 1996).

The Ca^{2+} Dependence of Calmodulin Binding to Syntrophin. The binding of syntrophin fusion proteins to dansyl-calmodulin was investigated in the presence and absence of calcium. Typical results are shown in Figure 6. Other calmodulin-binding proteins bind dansyl-calmodulin and alter its emission spectrum only in the presence of calcium but not in its absence (i.e., in the presence of EGTA) (Kincaid et al., 1982; Johnson & Wittenauer, 1983; Anderson et al., 1996). This typical behavior was observed for syntrophin B (Figure 6B). Since the only known calmodulin-binding sequence in syntrophin B is the CBS-C sequence, this region is most likely responsible for binding the Ca^{2+}-calmodulin. In contrast, syntrophin and syntrophin A bound dansyl-calmodulin and altered its fluorescence emission spectrum in either calcium or EGTA (Figure 6A,C). This calmodulin-binding resides in the syntrophin sequences since control fusion proteins (Mal F and His-Tag-β-galactosidase) lacking syntrophin sequences have no effect on dansyl-calmodulin's spectrum, and syntrophins I and G which do not bind calmodulin do not affect dansyl-calmodulin's emission spectrum (data not shown). From these data, we conclude that syntrophin can bind calmodulin in two different ways: amino-terminal syntrophin sequences (syntrophin A) can bind calmodulin in the presence or absence of Ca^{2+}, while binding to COOH-terminal syntrophin sequences (CBS-C of syntrophin B) is strictly Ca^{2+}-dependent.

These syntrophin sequences also bind biotinylated-calmodulin, as shown in Figure 7. In panel A, the binding of 250 nM biotinylated-calmodulin in the presence of 0.1 mM Ca^{2+} is shown. Syntrophin and fusion proteins containing either N-terminal (Syn A and E) or C-terminal (Syn B and

Table 2: Calmodulin-Binding Sequences

CBS-C Syntrophin[a]	
Mouse α1	PKtMVFIIHSFLSAKVTRLGLLA
Mouse α2	PKpIVFVLHTFLSAKVTRMGLLV
Human α1	PKtIVFIIHSFLSAKVTRLGLLA
Human β1	PKpIVFIIHSFLSAKITRLGLVA
Human β2	PKpIVFVLHTFLSAKVTRMGLLV
Rabbit α	PKtMVFIIHSFLSAKVTRLGLLA
	+ **A**+ A* +* +* **
CBS-1 Dystrophin Family[b]	
Human Dystrophin (18-42)[b]	KKTFTKWINAqFSKfGKqhIDNLFS
Mouse Dystrophin (18-42)	KKTFTKWINAqFSKfGKqhIDNLFS
Chicken Dystrophin (22-46)	KKTFTKWINAqFAKcGRrcIEDLFN
Human Utrophin (34-58)	KKTFTKWINArFSKsGKppINDMFT
	++ A +A* A + + * *A
CBS-2 Dystrophin Family	
Human Dystrophin (104-125)	HKLTLGLIWNIILHWQVKNVMK
Mouse Dystrophin (104-125	HKLTLGLIWNIILHWQVKNVMK
Chicken Dystrophin (108-129)	HKLTLGLIWNIILHWQVKDVMK
Human Utrophin (120-141)	HKLTLGLLWSIILHWQVKDVMK
	++* * **A ***+A *+ **+
CBS-3 Dystrophin Family	
Human Dystrophin (3325-3356)	RYRSLKHFNYDICQSCFFSGRVAKGHKMHYPM
Mouse Dystrophin (3318-3349)	RYRSLKHFNYDICQSCFFSGRVAKGHKMHYPM
Chicken Dystrophin (3322-3353)	RYRSLKHFNYDICQSCFFSGRVAKGHKMHYPM
Human Utrophin (3082-3113)	RYRSLKHFNYDVCQSCFFSGRTAKGHKLHYPM
	+A+ *++A A-* AA + + ++*+A *
CBS Myosin Light Chain Kinase (Skeletal)[c]	
Rabbit MLCK (577-603)	KRRWKKNFIAVSAANRFKKISSSGALM
	+++A++ A* * +A++* **

[a] Genebank Accession Numbers are U00677, U00678, U40571, L31529, U40572, and U01243 for mouse α1, α2, human α1, β1, β2, and rabbit α syntrophins, respectively. For each gene family shown in this table, positions identical in all sequences are shown in upper case, bold type while those which are homologous are shown as upper case only. The chemical properties of each sequence is also depicted using "+", "−", "*", and "A", to denote basic, acidic, aliphatic hydrophobic, and aromatic hydrophobic residues, respectively. [b] Dystrophin CBS-1 and -2 are from Jarrett and Foster (1995) while the CBS-3 sequences are the dystrophin exon 69 sequences reported in Anderson et al. (1996). Dystrophin Genebank Accession Numbers are M18533, M68859, and X13369 for human, mouse, and chicken respectively; human utrophin is X69086. [c] Shown is the M13 calmodulin-binding peptide (Takio et al., 1986).

F) bind calmodulin, while His-Tag-β-galactosidase (expressed and purified in the same way) does not and provides a negative control (Figure 7A). In related experiments, the Mal F (panel B) or protein induced and purified from cells containing the pET28 plasmid without a syntrophin DNA insert also fail to bind the biotinylated-calmodulin (data not shown), demonstrating that binding is derived from the syntrophin sequences. Binding occurs in both the amino-terminal (Syn A, Figure 7A) and carboxy-terminal (Syn B) ends of syntrophin, and the carboxy-terminal binding occurs even with Syn F which contains only the 24 residue CBS-C sequence. Panel B shows that biotinylated-calmodulin binding to syntrophin also occurs when EGTA replaces the Ca^{2+}, albeit to a lesser extent.

There is clearly a dilemma posed by these experiments: if syntrophin binds calmodulin in EGTA (Figures 6 and 7), why does it elute from calmodulin−Sepharose in EGTA (Figure 4)? By answering this question, we have discovered previously unknown properties of syntrophin.

Why Does Syntrophin Elute from Calmodulin−Sepharose? The simplest explanation would be that much of the applied syntrophin did not elute in EGTA and remains bound by the column in Figure 4. This was investigated by applying syntrophin and syntrophin A to both Sepharose and calmodulin−Sepharose columns of equal size and determining the amount of protein recovered. Both syntrophin fusion proteins pass through the Sepharose column unretained (data not shown) while on calmodulin−Sepharose a portion is retained and elutes in EGTA (Figure 4). From either column, 93−100% of the syntrophin or syntrophin A applied was recovered after chromatography. Thus, although syntrophin can interact with calmodulin in EGTA (Figures 6 and 7), syntrophin elutes completely from calmodulin−Sepharose in EGTA (Figure 4).

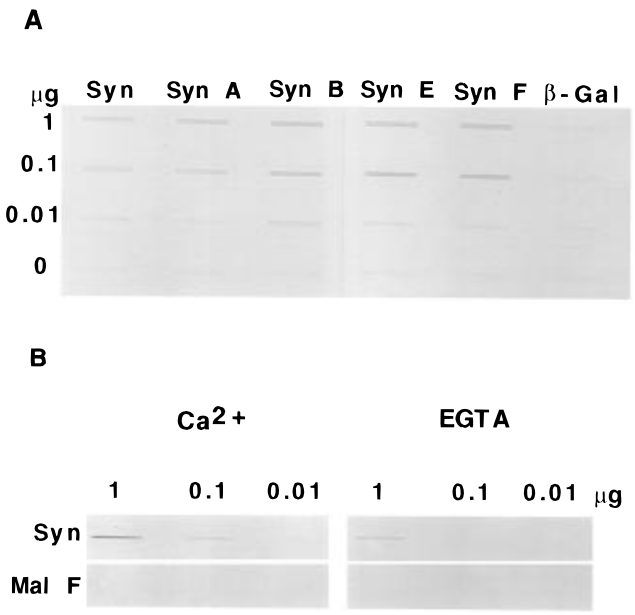

FIGURE 7: Syntrophin also binds biotinylated-calmodulin. Syntrophin fusion proteins were blotted onto nitrocellulose (μg blotted are shown on the figure) and probed with 250 nM biotinylated-calmodulin using procedures previously described (Jarrett & Madhavan, 1991). Syntrophin (Syn) and β-galactosidase (β-Gal) were both His-Tag fusion proteins; all others were MBP fusions. Panel A, biotinylated-calmodulin binding when 0.1 mM $CaCl_2$ was present throughout the procedure in all buffer solutions. Panel B, 0.1 mM EGTA replaced $CaCl_2$ during biotinylated-calmodulin binding and the three subsequent washes (right hand side), but otherwise the procedure was the same as panel A.

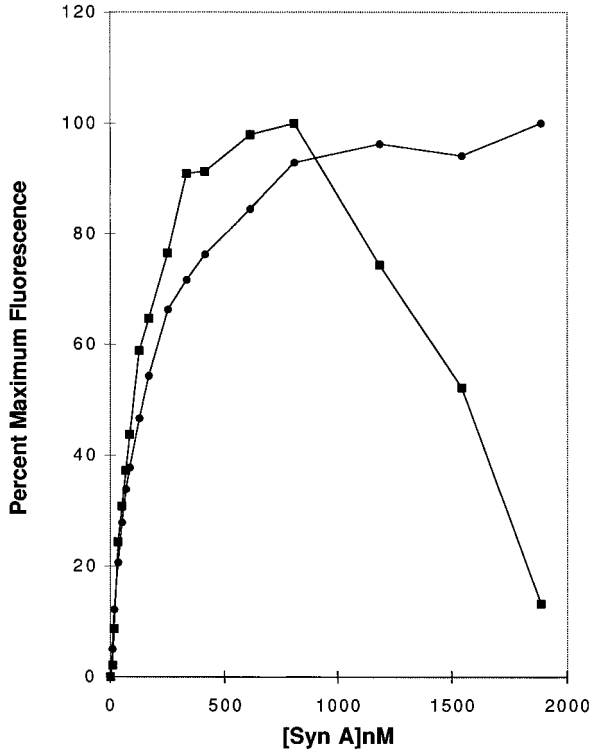

FIGURE 8: The binding of syntrophin A to dansyl-calmodulin. See the legend to Figure 5 for further details. The fluorescence of dansyl-calmodulin was monitored (excitation 345, emission 490) as His-Tag-syntrophin A was added. The buffer contained 0.1 mg/mL ovalbumin and either 1 mM Ca^{2+} (●, buffer MC) or 1 mM EGTA (■, buffer ME). An identical titration in the absence of dansyl-calmodulin was subtracted from the data, and the data were scaled so that maximum observed fluorescence was 100%.

Another possible explanation would require that calmodulin binding in EGTA is of much lower affinity. An experiment to test this is shown in Figure 8. On the left side of the figure, at lower syntrophin A concentrations, half-maximal binding of dansyl-calmodulin occurs at ~0.1 μM whether Ca^{2+} or EGTA was present. Thus, syntrophin A binds calmodulin equally well, independent of Ca^{2+}. The main difference brought about by Ca^{2+} occurs at the high syntrophin A concentrations found on the right side of the figure; in the absence of Ca^{2+}, some process occurring at high syntrophin A concentrations is markedly inhibiting calmodulin binding (Figure 8). Since the concentration of syntrophin A used in the calmodulin−Sepharose experiments (0.2 mg/mL, 6.2 μM) is in the micromolar range where this inhibition of calmodulin binding occurs (Figure 8), this phenomenon accounts for elution from calmodulin−Sepharose by EGTA (Figure 4). Our hypothesis, based upon these results, is that, in the absence of Ca^{2+}, syntrophin oligomerizes if concentrations are sufficiently high, and this oligomerization blocks amino-terminal calmodulin binding. This hypothesis will be discussed further below.

Another consequence of Figure 8 is that syntrophin must bind Ca^{2+}. Since dansyl-calmodulin binds equally well in the presence or absence of Ca^{2+} at low syntrophin concentrations, the Ca^{2+}-binding properties of calmodulin cannot explain the inhibition observed in EGTA at higher syntrophin concentrations (Figure 8). Rather, Ca^{2+} must affect syntrophin directly, and thus syntrophin must bind Ca^{2+}.

Syntrophin Is a Ca^{2+}-Binding Protein. To test this, a limited equilibrium dialysis experiment was performed to determine whether syntrophin A or B binds Ca^{2+}. Using different preparations of protein for each experiment, two separate experiments were performed at 50 μM Ca^{2+} and the stoichiometries averaged. The results are that syntrophin A binds 2.1 ± 0.2 mol of Ca^{2+}/mol while syntrophin B binds 0.2 ± 0.1 (data not shown).

Syntrophin A has four tryptophan residues which could serve as intrinsic fluorescent probes to assess Ca^{2+} binding. As shown in Figure 9A, the fluorescent emission of His-Tag-syntrophin A tryptophan is quenched upon the addition of Ca^{2+}. The uppermost spectrum is that of the Ca^{2+}-free protein (0.5 mM EDTA), and as greater amounts of Ca^{2+} are added in the range of 0.5−2.5 mM, the emission is progressively diminished. The effect is progressive and saturable. It should be noted that total Ca^{2+} concentrations (in the presence of 0.5 mM EDTA) are given in the figure legend. In the case of 0.5 mM Ca^{2+} and 0.5 mM EDTA (the second highest emission spectrum, panel A), the calculated concentration of free (uncomplexed) Ca^{2+} is 10 μM. This concentration gave 43% of the maximum quenching observed (at 2.5 mM total Ca^{2+}). Thus, in agreement with the equilibrium dialysis result, syntrophin binds significant Ca^{2+} even at concentrations in the micromolar range. MBP−syntrophin E, which contains nearly full-length syntrophin sequences (see Figure 1), behaves similarly (Figure 9B). These results, taken together with the equilibrium dialysis results, demonstrate that Ca^{2+} binding is an attribute of syntrophin sequence contained in syntrophins A and E but absent in syntrophin B and that binding is not due to the fused sequences.

To observe its oligomeric state, His-Tag-syntrophin A (16.9 μM) was subjected to size exclusion chromatography

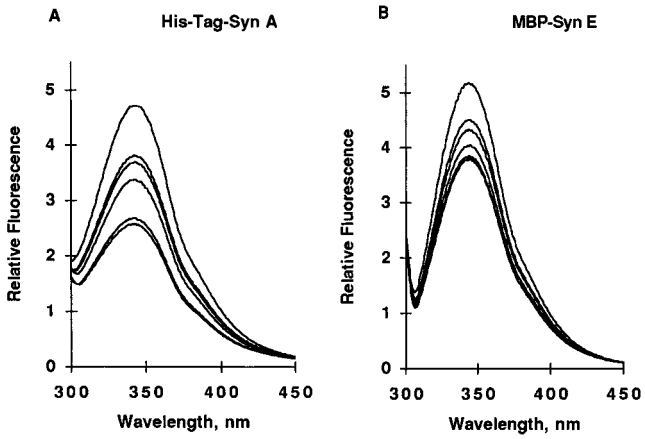

FIGURE 9: Ca^{2+} affects the intrinsic tryptophan fluorescence emission of syntrophin. His-Tag-syntrophin A (panel A) or MBP-syntrophin E (panel B) were dialyzed into buffer PE (10 mM PIPES, pH 6.5, 100 mM KCl, 0.5 mM EDTA). One gram of Chelex was added to the solution outside the dialysis bag. After dialysis, samples were filtered (0.45 μm) and adjusted to either 10 μM (syntrophin A) or 2 μM (syntrophin E) in buffer PE. Excitation was 280 nM, and emission was recorded from 300 to 450 nm; both slits were set at 16 nm bandpass. Additions of 100 mM $CaCl_2$ were made, and the final resulting concentrations were (in decreasing order from the top spectrum): 0 (EDTA only), 0.5, 1, 1.5, 2, or 2.5 mM $CaCl_2$ for Syn A (panel A) and 0, 1, 1.5, 2, 2.5, and 3 mM for Syn E (panel B).

in an EGTA containing buffer. Syntrophin A gives two peaks which, when compared to molecular mass standards, have apparent molecular mass of 29 and 61 kDa (data not shown but submitted for review). The actual molecular mass of His-Tag-syntrophin A is 32.5 kDa, and thus these two peaks are near the size expected for syntrophin A monomer and dimer. The two peaks are not of equal size. The larger peak (67% of total peak area) is the dimer.

DISCUSSION

This investigation began with the limited objective of determining which syntrophin sequences bind calmodulin and whether this affects dystrophin binding. Although only partially successful, it led to several unexpected discoveries about syntrophin which are potentially important.

Syntrophin binding to dystrophin C-terminal sequences is antagonized by Ca^{2+}-calmodulin *in vitro* (Figure 3). Calmodulin also inhibits the actin−dystrophin interaction which occurs at the amino-terminus of dystrophin (Jarrett & Foster, 1995). Whether calmodulin modulates either interaction *in vivo* is not known. Where Ca^{2+}-calmodulin binds to antagonize the syntrophin interaction is also not clear presently. Dystrophin also binds calmodulin, and one of these binding sites, CBS-3, is present in the DysS9 fusion protein (Anderson et al., 1996). Since syntrophin also binds calmodulin, calmodulin may bind to syntrophin, dystrophin, or both to cause the observed inhibition. Dystrophin's CBS-3 has an apparent affinity for calmodulin near 54 nM (Anderson et al., 1996), while syntrophin's calmodulin binding has apparent affinities of about 18 nM (Syn B) and 98 nM (Syn A). The measure K_I for Ca^{2+}-calmodulin, 343 nM, corresponds to none of these values, suggesting that inhibition may be quite complex in spite of the good fit obtained with a simple competitive inhibition model.

Calmodulin binding to syntrophin is complex; a schematic representation of this binding is presented in Figure 10.

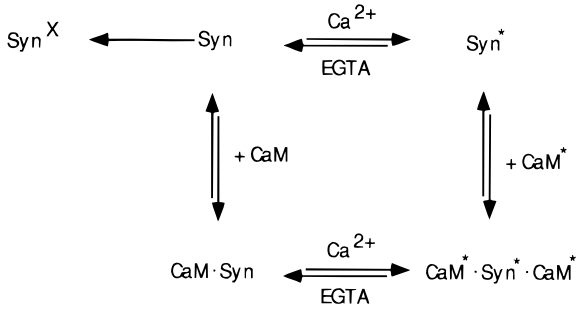

FIGURE 10: A model depicting calmodulin binding to different forms of syntrophin. In this model, the calcium bound forms of calmodulin and syntrophin are denoted with an asterisk (*). The form of syntrophin which occurs at high syntrophin concentrations in the absence of Ca^{2+} is indicated as Syn^X.

Calmodulin binds to syntrophin in at least two different ways: Ca^{2+}-calmodulin (denoted with an asterisk) and apocalmodulin (i.e., Ca^{2+}-free calmodulin) bind to syntrophin amino-terminal sequences (indicated by binding to the left of Syn), while only Ca^{2+}-calmodulin binds to the CBS-C in syntrophin's unique COOH-terminal domain (shown to the right of Syn). Syntrophin's highest affinity calmodulin binding derives primarily from these CBS-C sequences (Table 1). CBS-C is highly conserved in all syntrophins and among animal species as are the calmodulin-binding sites of dystrophin and utrophin (Table 2).

The calmodulin binding which occurs in syntrophin's amino-terminal sequences (Figure 10) was only partially localized to syntrophin sequences 4−173 present in the syntrophin H fusion protein (see Figures 1 and 4). The binding measured with dansyl-calmodulin is of about 5-fold lower affinity than binding to CBS-C (Table 1). While binding occurs in the absence of Ca^{2+} (Figures 6A,C, 7B, and 8), it is inhibited at higher syntrophin concentrations in EGTA (Figure 8). It is because of this latter phenomenon that complete elution from calmodulin−Sepharose (Figure 4) occurs in EGTA. We have discussed this phenomenon as potentially involving syntrophin oligomerization, but any process which alters calmodulin binding at high syntrophin concentrations would be consistent with the data presented and thus we depict this form as Syn^X (Figure 10).

Oligomerization appears a likely explanation for several reasons. Inhibition occurs only at high protein concentrations (Figure 8) and oligomerization would be highly dependent upon protein concentration. α1 Syntrophin has been shown to bind all three (α1, β1, and β2) DGC syntrophin bands in gel blot overlay experiments (Madhavan & Jarrett, 1995a). Thus, syntrophins must have the capacity to form at least dimers. We have shown here that syntrophin A exists predominantly as a dimer in EGTA. Dimerization may not be the limit of oligomerization since we have found that high concentrations of syntrophin and syntrophin A form visible aggregates under some conditions which can be pelletted readily by low speed centrifugation (unpublished data). This may be related to the aggregation reported for α1 and β2 syntrophin by Ahn et al. (1996). In our experience, aggregation did not occur if syntrophin's were stored at concentrations less than 1 mg/mL in 15% glycerol (see Experimental Procedures) and dialyzed only as dilute solutions. Thus, oligomerization does occur but whether this is responsible for diminishing apocalmodulin binding (Figure 8) has not been firmly established.

An interesting feature of the scheme presented in Figure 10 is the linkage which would occur between calmodulin binding and the process (e.g., oligomerization) which makes syntrophin refractive to binding (Syn^X). The scheme predicts that calmodulin at high levels would inhibit this process, although this has not yet been tested. Calmodulin does bind to phosphofructokinase to prevent formation of the active tetramer of that enzyme *in vitro* (Buschmeier et al., 1987), and recently, calmodulin has been shown to alter the oligomeric state and activity of smooth muscle myosin light chain kinase (Sobieszek et al., 1993; Babiychuk et al., 1995). Other proteins also bind syntrophin including utrophin and dystrophin (Kramarcy et al., 1994), neuronal-type nitric oxide synthase (Brenman et al., 1996), and α- and γ-sarcoglycan (Madhavan & Jarrett, 1995a). The scheme in Figure 10 suggests that, depending upon whether binding occurs to aposyntrophin, Ca^{2+}-syntrophin, or some particular oligomeric form of syntrophin, binding could potentially be regulated by Ca^{2+} or calmodulin. Such regulation was demonstrated for the binding of dystrophin's COOH-terminus to syntrophin (Figure 3).

Whatever process is involved, it occurs in the absence of Ca^{2+} (Figure 8). This requires that syntrophin bind Ca^{2+} directly, and this was confirmed by equilibrium dialysis and in a fluorescence experiment (Figure 9). Since sufficient materials were available for only a limited investigation, little is known about the affinity for calcium or the maximum number of Ca^{2+}-binding sites. However, at 50 μM Ca^{2+}, the stoichiometry was 2.2 mol/mol, showing that more than one binding site is present and binding must be of reasonably high affinity.

Syntrophin's sequence was searched for the "EF hand" calcium-binding motif (Kretsinger, 1980) without finding a good match. This EF hand motif accounts for the high affinity Ca^{2+} binding of calmodulin and many other proteins; Ca^{2+}-binding sites have also been identified in some pleckstrin homology domains (Tyer et al., 1988; Mahadevan et al., 1995) of which syntrophin has two (Gibson et al., 1994). It is interesting to note that the Ca^{2+}-binding site located in the PH domain of the protooncogene *dbl* has recently been shown to be functional and to bind Ca^{2+} with an apparent affinity of 10 μM (Mahadevan et al., 1995). Although the match was not impressive, the COOH-terminal 2/3 of the PH1b domain of syntrophin contains the two best matches to the EF hand motif and these may participate in Ca^{2+} binding. However, high affinity Ca^{2+} binding to thermolysin and other proteins occurs utilizing non-EF hand binding sites, and this appears to also be the case with syntrophin. How syntrophin binds Ca^{2+} and whether Ca^{2+} is the preferred ligand for these sites await further investigation.

The binding of apocalmodulin to proteins has precedents. Although calmodulin typically binds its target sequences in the presence of calcium, calcium-independent binding has been observed in several instances which fall into two recognized classes. An example of the first type is calmodulin binding to the catalytic (γ) subunit of phosphorylase kinase. This binding occurs in the absence of calcium and apparently results from concerted binding of calmodulin to two noncontiguous domains (Dasgupta et al., 1989). These two domains (PhK 5 and 13) are also cationic, amphipathic sequences, and it is the concerted binding of both which apparently results in the association of calmodulin even in the absence of calcium. Thus, this kind of calcium-independent binding is similar to calcium-dependent binding in the sequences bound but differs in the concerted interaction with multiple sites. This kind of binding may also describe the calmodulin binding to syntrophin 4−173 since cationic amphipathic sequences occur within this sequence region (e.g., syntrophin 77−86 and 96−115). These sequences are in the PDZ domain of syntrophin. The other kind of calcium-independent binding involves the IQ domain motif (Cheney & Mooseker, 1992). Multiple tandem copies of the IQ motif (IQXXXRGXXXR, where X is any amino acid) are found in non-muscle myosin and bind the regulatory light chains (Houdusse & Cohen, 1995); these myosin light chains are homologous to calmodulin, troponin C, and other members of this gene family. All currently known syntrophin sequences were searched without success for this motif. Thus, syntrophin's N-terminal calmodulin binding occurs either by the first kind of concerted binding or by some currently unknown type of interaction.

This study has resulted in several important new observations about syntrophin. Syntrophin's interaction with dystrophin's COOH-terminal domain is inhibited by Ca^{2+}-calmodulin. Syntrophin binds calmodulin at both ends of its sequence. The COOH-terminal Ca^{2+}-calmodulin binding occurs at syntrophin 480−503, is similar to other known calmodulin-binding sites, and is highly conserved. This calmodulin-binding site makes up about half of the SU domain, the unique domain found only in syntrophins. The amino-terminal binding was not precisely located but occurs within syntrophin 4−173 sequences, and calmodulin binds this region of sequence even in the absence of Ca^{2+}. Finally, syntrophin itself is a Ca^{2+}-binding protein. All of these are new and previously unknown.

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