# Determination of the Oligomeric Status of the Sarcoplasmic Reticulum $Ca^{2+}$-ATPase Using Optimized Chemical Crosslinking

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**Abstract**

In determining protein-protein interactions in native muscle membranes biochemically, bifunctional crosslinking agents of various length and solubility have been established as effective tools. Gel electrophoretic and immunoblotting methodology have proven to be highly suitable for the analysis of crosslinked products. Here, we have analysed the oligomeric status of a well established muscle membrane complex, the sarcoplasmic reticulum $Ca^{2+}$-ATPase, employing a rapid optimization procedure of chemical crosslinking. Both, the fast SERCA1 and the slow SERCA2 isoforms of this enzyme appear to exist predominantly as a tetrameric complex under native conditions. Direct protein-protein interactions are postulated to be important for cooperative kinetics and protection against proteolytic degradation. In order to keep artifacts of random crosslinking and hydrolysis of crosslinkers to a minimum, an efficient and swift experimental scheme is described to improve reaction conditions with respect to concentration ratios between crosslinkers and biomembranes, length of incubation time, pH and temperature. Employing the 11.4-Å probe bis-sulfosuccinimidyl-suberate and a mini-gel system for the analysis of microsomal vesicles, highly reproducible and optimal results could be obtained with relatively small amounts of skeletal muscle tissue. Thus, the reaction scheme of optimization of crosslinking described in this study is generally suitable for the analysis of supramolecular complexes in biomembranes and should improve the initial determination of the quaternary protein structures within muscle membrane microdomains.

**Key words**: bissulfosuccinimidyl-suberate, $Ca^{2+}$-ATPase, chemical crosslinking, oligomerization, sarcoplasmic reticulum.

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It is generally acknowledged that many of the key membrane components involved in $Ca^{2+}$-regulatory processes in skeletal muscle fibres exist in high-molecular-mass complexes [29, 31]. This includes key components of excitation-contraction coupling and $Ca^{2+}$-sequestration such as the voltage-sensing dihydropyridine receptor of the transverse tubules, the ryanodine receptor $Ca^{2+}$-release channel of the junctional sarcoplasmic reticulum, and the high-capacity $Ca^{2+}$-binding protein calsequestrin of the terminal cisternae region [31-33]. In addition, one of the enzyme systems which plays a central role in $Ca^{2+}$-homeostasis and facilitation of muscle relaxation, the fast and slow isoforms of the sarcoplasmic or endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) [22], was also found to exist as a large membrane complex [24]. The monomeric form of this relatively abundant $Ca^{2+}$-pump has an apparent molecular mass of 110 kDa and its molecular structure is predicted to contain a stalk domain and a transmembrane domain, as well as a cytoplasmic head piece with the functionally important nucleotide binding site and phosphorylation domain [23]. The complex reaction cycle of this integral protein involves the formation of a phosphoprotein intermediate and provides the active pumping of two $Ca^{2+}$-ions from the sarcoplasm into the lumen of the sarcoplasmic reticulum at the expense of one molecule of adenosine triphosphate being hydrolysed [26]. Recent structural studies at 8-Å resolution determined a distinct cavity within SERCA molecules, which is believed to lead to the putative $Ca^{2+}$-binding site, thereby providing the path for $Ca^{2+}$-ions into the lumen of the sarcoplasmic reticulum [42]. Since SERCA pump units form 8.5 nm intramembraneous particles, it was suggested that the native $Ca^{2+}$-ATPase exists as a tetrameric structure [27].

Direct protein-protein interactions are proposed to be essential for proper physiological functioning of $Ca^{2+}$-ATPase units and complex formation may be involved in cooperative kinetics and protection against proteolytic degradation [26].

Chemical crosslinking is a widely used biochemical technique both for the analysis and stabilization of multi-molecular aggregates in biological membranes [8, 10, 12, 15, 30, 35, 40]. Both homo- and hetero-bifunctional reagents are in common use for the elucidation of the quaternary structure of oligomeric proteins and their native organization in membrane systems [9, 13, 16, 28, 38]. In addition, photolabeling of biomolecules is a widespread analytical method in determining sensitive protein-protein interactions [7]. In conjunction with biochemical information on primary peptide structure, biophysical data on the secondary and tertiary arrangement of protein domains and cryo-electron microscopical knowledge about the three-dimensional organization of proteins, chemical crosslinking can be used as a highly reliable tool in confirming close neighborhood relationships between membrane proteins. However, since the interactions between chemical crosslinkers and biomembranes might introduce potential artifacts, it is absolutely imperative that controlled conditions with respect to concentration ratios between membrane proteins and crosslinkers, buffer composition, temperature, pH and length of incubation time be employed [39].

In this study we describe a fast and efficient experimental procedure for optimizing these critical conditions in order to minimize random crosslinking in the analysis of muscle protein complexes in native membranes. To exclude potential complications with a low rate of non-specific exchange between previously uncrosslinked proteins containing a free thiol group and specifically crosslinked proteins, we avoided the use of a reagent containing cleavable disulfide bridges. Instead, we performed our analysis with the 11.4-Å probe bis-sulfosuccinimidylsuberate $(BS^3)^2$ [17], which induces covalent bonds that are not sensitive to chemical reduction. Hence, gel electrophoretic separation could be carried out under reducing conditions, which was advantageous for the subsequent immunoblotting step since many antibodies recognize the reduced form of epitopes better than non-reduced domains.

## Materials and Methods

### *Materials*

The chemical crosslinker bis-sulfosuccinimidyl suberate, as well as chemiluminescence substrates were purchased from Pierce and Warriner Limited (Chester, UK). Monoclonal antibodies IIH11 and IID8 to the fast-twitch SERCA1 isoform and the slow-twitch SERCA2 isoform of the sarcoplasmic reticulum $Ca^{2+}$-ATPase, respectively, were obtained from Affinity Bioreagents (Golden, CO). Acrylamide stock solutions, protease inhibitors and peroxidase-conjugated secondary antibodies were from Boehringer-Mannheim (Lewis, UK). Immobilon NC nitrocellulose membranes were obtained from Millipore Corporation (Bedford, MA). All other chemicals were of analytical grade and purchased from Sigma Chemical Company (Dorset, UK).

### *Isolation of muscle membrane vesicles*

Rabbit back and leg skeletal muscle, obtained from the Biomedical Facility, National University of Ireland, Dublin was homogenized in 7.5 volumes of 25 mM Hepes, 10% (w/v) sucrose, 1 mM EGTA, 0.02% (w/v) sodium azide, 3 mM $MgCl_2$ and crude microsomal membranes were prepared as previously described in detail [32, 33]. All buffers contained a mixture of protease inhibitors (0.2 mM Pefabloc, 1.4 mM pepstatin A, 0.3 mM E-64, 1 mM leupeptin, 1 mM EDTA, 0.5 mM soybean trypsin inhibitor) [32] and all preparative steps were performed at 0-4°C. Protein concentration was determined according to Bradford [5] using myofibrillar proteins as a standard. Microsomal vesicles were resuspended in buffer (containing a fresh protease inhibitor cocktail) at a protein concentration of 10 mg/ml and then used immediately for chemical crosslinking experiments.

### *Chemical crosslinking analysis*

The non-cleavable, membrane-impermeable 11.4-Å crosslinker bissulfosuccinimidyl suberate ($BS^3$) was used for chemical crosslinking of muscle membrane proteins [20, 32, 33]. In order to minimize hydrolysis in an aqueous environment, $BS^3$ was dissolved in 20 mM citrate buffer, pH 5.0 at a stock concentration of 5 mg/ml. For the initial determination of the optimum ratio between the bifunctional reagent and membrane proteins, vesicles were incubated for 30 min at 21°C with 1 to 500 mg crosslinker per mg membrane protein in 50 mM Hepes, pH 8.0. Subsequently, the chemical crosslinking reaction was terminated by the addition of 50 ml of 1 M ammonium acetate per ml of reaction mixture [32] and an equal volume of sodium dodecyl sulfate-containing sample buffer [19] was added to the suspension. The mixture was warmed for 10 min at 37°C and then proteins were electrophoretically separated [19]. Following the determination of the optimum crosslinker concentration by immunoblot analysis [37], membrane vesicles were incubated with $BS^3$ at a range of temperatures (0°C, 4°C, 10°C, 21°C, 37°C) pH-values (pH 5.0, pH 6.0, pH 7.0, pH 8.0, pH 9.0, pH 10.0) and incubation times (30 s, 1 min, 5 min, 10 min, 15 min, 30 min, 45 min).

### *Gel electrophoresis and immunoblotting*

Using a Bio-Rad Mini-Protean II gel system (Bio-Rad Laboratories, Hemel Hempstead, Herts., UK), standard polyacrylamide gel electrophoretic separation of

proteins under reducing conditions in the presence of sodium dodecyl sulfate was performed according to Laemmali [19]. 7% (w/v) polyacrylamide gels were run for 280 Vh with 30 mg protein per lane. Rat myofibrillar proteins served as an established source for high-molecular-mass standards [32]. The method of Towbin et al. [37] was used to electrophoretically transfer crosslinked proteins to nitrocellulose paper using a Bio-Rad Mini-Protean II blotting system (Bio-Rad Laboratories, Hemel Hempstead, Herts., UK). Blocking of nitrocellulose sheets, as well as incubation with primary and secondary antibodies was performed as previously described [32] and visualization of immuno-reactivity was achieved by enhanced chemiluminescence (ECL) [4]. Densitometric scanning of ECL-blots was performed on a Molecular Dynamics 300S computing densitometer (Sunyvale, CA) using ImageQuant V3.0 software. The data is expressed relative to immuno-decorated protein bands *(see* Fig. 1b, lane 1) displaying maximum labeling using the ECL method.

## Results

Besides ultracentrifugation and gel-filtration chromatography [39], electrophoretic separation methodology in combination with immunoblotting has proven to be extremely useful in the analysis of multi-molecular aggregates following chemical crosslinking. In this study we outline a fast optimization procedure for chemical crosslinking of a muscle membrane complex. Prior to the crosslinking experiments presented here, various agents with different reactivity, spacer-arm length, specificity and solubility were tested, as has been previously described in detail [11, 20, 24, 31-33]. Once $BS^3$ had been determined to represent a suitable crosslinker for the analysis of the SERCA1 isoform of the sarcoplasmic reticulum $Ca^{2+}$-ATPase, optimum concentration ratios between the crosslinker and the membrane protein were investigated.

### *Chemical crosslinking analysis of SERCA1 $Ca^{2+}$-ATPase*

As illustrated in the representative Coomassie-stained protein gel and immunoblot in Fig. 1a, b, testing a range of 1 to 500 mg $BS^3$ per mg protein was useful as an initial analytical step. At 10 to 200 mg crosslinker per mg protein, a decrease in the apparent 110 kDa monomer of SERCA1 and the concomitant appearance of a highmolecular-mass band was observed. The immuno-decorated bands of decreased relative electrophoretic mobility may represent tetrameric structures of the $Ca^{2+}$-pump. Since maximum oligomerization was achieved at 50 and 100 mg crosslinker per mg membrane protein (Fig. 1c), we performed the subsequent optimization experiments with the lower optimal crosslinker to protein ratio.

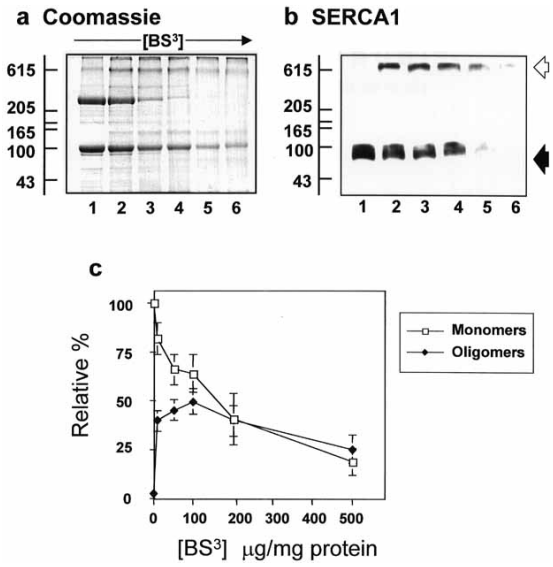


*Figure 1. Chemical crosslinking analysis of the SERCA1 isoform of the $Ca^{2+}$-ATPase from rabbit skeletal muscle. Shown is a Coomassie-stained gel (a) and an identical nitrocellulose blot (b) immuno-decorated with monoclonal antibody IIH11 against the fast-twitch $Ca^{2+}$-ATPase. Lanes 1 to 6 represent 1, 10, 50, 100, 200, and 500 mg crosslinker ($BS^3$) per mg membrane protein, respectively. Microsomes were incubated for 30 min at 21°C using pH 8.0. The sizes of molecular mass standards (in kDa) are indicated on the left. The relative positions of apparent monomers and crosslinked oligomers are marked by solid and open arrows, respectively. The relative amount of immunodecorated monomers and oligomers as evaluated by densitometry of ECL blots (n=5) is depicted in panel (c).*

### *Effect of incubation time on SERCA1 crosslinking*

Investigating reaction times ranging from 30 seconds to 45 minutes, it was determined that 10 to 30 minutes of incubation of rabbit skeletal muscle microsomes with 50 mg $BS^3$ per mg protein resulted in the optimum appearance of immuno-decorated high-molecular-mass bands representing SERCA1 complexes (Fig. 2b, c). In analogy, Coomassie-stained protein gels also showed a new band of decreased electrophoretic mobility at these incubation times (Fig. 2a). Reaction times less than 5 minutes resulted in much less oligomerization of the $Ca^{2+}$-ATPase (Fig. 2b, c). In addition to the protein band of approximately 630 kDa, a second high-molecular-mass band was observed for the samples incubated for longer than 5 minutes at the interface between the stacking gel and the separation gel (Fig. 2b). These immuno-decorated bands might represent very large aggregates of the $Ca^{2+}$-pump which can not be properly

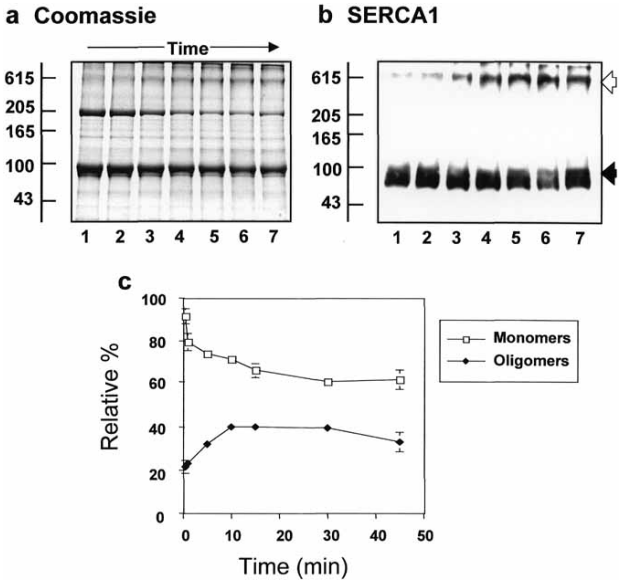


*Figure 2. Effect of incubation time on chemical crosslinking of the SERCA1 isoform of the $Ca^{2+}$-ATPase from rabbit skeletal muscle. Shown is a Coomassie-stained gel (a) and an identical nitrocellulose blot (b) immunodecorated with monoclonal antibody IIH11 against the fast-twitch $Ca^{2+}$-ATPase. Lanes 1 to 7 represent the incubation of microsomes at pH 8.0 and 21°C with 50 mg crosslinker ($BS^3$) per mg membrane protein for 0.5, 1, 5, 10, 15, 30 and 45 minutes, respectively. The sizes of molecular mass standards (in kDa) are indicated on the left. The relative positions of apparent monomers and crosslinked oligomers are marked by solid and open arrows, respectively. The relative amount of immunodecorated monomers and oligomers as evaluated by densitometry of ECL blots (n=5) is depicted in panel (c).*

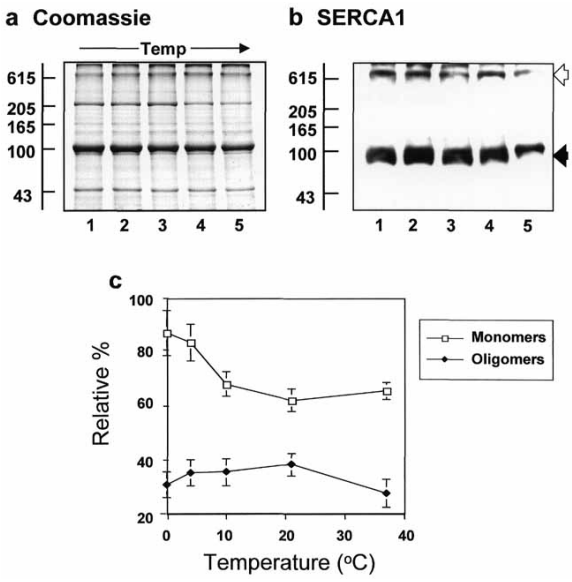


*Figure 3. Effect of temperature on chemical crosslinking of the SERCA1 isoform of the $Ca^{2+}$-ATPase from rabbit skeletal muscle. Shown is a Coomassie-stained gel (a) and an identical nitrocellulose blot (b) immunodecorated with monoclonal antibody IIH11 against the fast-twitch $Ca^{2+}$-ATPase. Lanes 1 to 5 represent the incubation of microsomes for 30 min and pH 8.0 with 50 mg crosslinker ($BS^3$) per mg membrane protein at O°C, 4°C, 10°C, 21°C, and 37°C, respectively. The sizes of molecular mass standards (in kDa) are indicated on the left. The relative positions of apparent monomers and crosslinked oligomers are marked by solid and open arrows, respectively. The relative amount of immunodecorated monomers and oligomers as evaluated by densitometry of ECL blots (n=5) is depicted in panel (c).*

separated by the polyacrylamide gel electrophoresis system.

*Effect of temperature on SERCA1 crosslinking*

In contrast, variations in temperature had a lesser effect on the outcome of the crosslinking reaction than differing incubation times. The Coomassie gel in Fig. 3a did not reveal significant differences in the overall protein band pattern of the microsomal fraction incubated at 0 to 37°C. With the exception of the highest temperature investigated, incubation temperatures ranging from 0 to 21°C resulted in very comparable oligomerization patterns for the $Ca^{2+}$-ATPase (Fig. 3b, c). Thus, incubation with 50 mg $BS^3$ per mg membrane protein can be performed both on ice or at room temperature depending on the experimental conditions needed for subsequent analytical purposes.

*Effect of pH on SERCA1 crosslinking*

Very interesting results were obtained with crosslinking experiments at varying pH (Fig. 4). As shown in the immunoblot of Fig. 4b, at pH 7.0 a SERCA1 band representing apparent dimers was immunolabeled as well as high-molecular-mass bands representing apparent tetramers. This phenomena was not observed at lower or higher pH-values investigated (Fig. 4c). Hence, the pH and buffering system might have a profound effect on the outcome of the crosslinking pattern and it is recommended to test more than one pH-value in the analysis of protein-protein interactions in native muscle membrane systems.

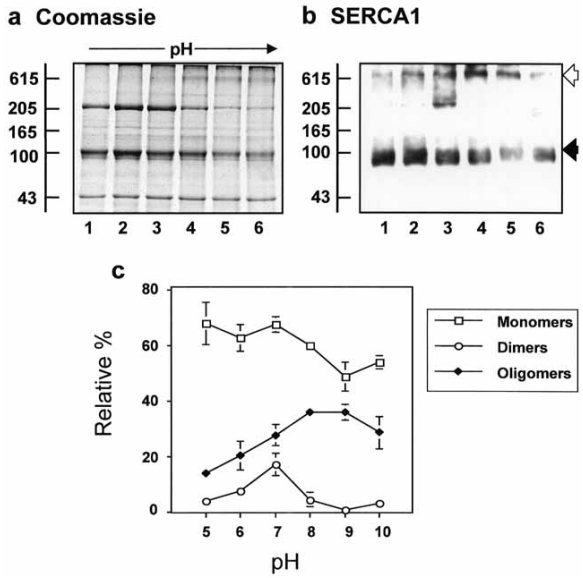


*Figure 4. Effect of pH on chemical crosslinking of the SERCA1 isoform of the $Ca^{2+}$-ATPase from rabbit skeletal muscle. Shown is a Coomassie-stained gel (a) and an identical nitrocellulose blot (b) immunodecorated with monoclonal antibody IIH11 against the fast-twitch $Ca^{2+}$-ATPase. Lanes 1 to 6 represent the incubation of microsomes for 30 min a 21°C with 50 mg crosslinker ($BS^3$) per mg membrane protein using pH 5.0, pH 6.0, pH 7.0, pH 8.0, pH 9.0, and pH 10.0, respectively. The sizes of molecular mass standards (in kDa) are indicated on the left. The relative positions of apparent monomers and crosslinked oligomers are marked by solid and open arrows, respectively. The relative amount of immunodecorated monomers and oligomers as evaluated by densitometry of ECL blots (n=5) is depicted in panel (c).*

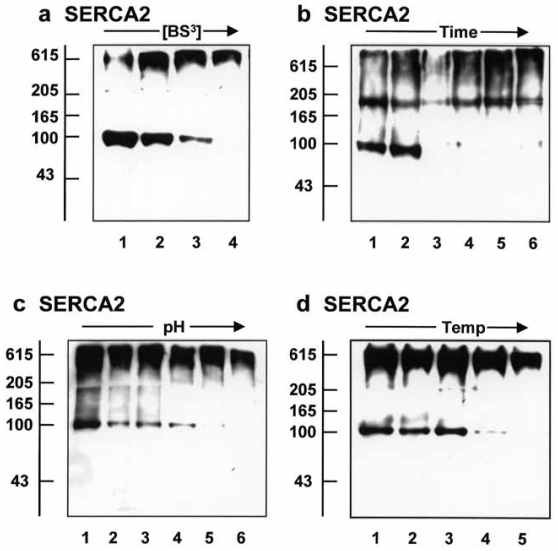


*Figure 5. Effect of crosslinker concentration, incubation time, pH, and temperature on chemical crosslinking of the slow SERCA2 isoform of the $Ca^{2+}$-ATPase from rabbit skeletal muscle. Shown are nitrocellulose blots immuno-decorated with monoclonal antibody IID8 against the slow-twitch $Ca^{2+}$-ATPase. In (a), lanes 1 to 4 represent the incubation of microsomes for 30 min at 21°C using pH 8.0 with 0, 12.5, 25, and 50 mg crosslinker ($BS^3$) per mg membrane protein, respectively. In (b), lanes 1 to 6 represent the incubation of microsomes at pH 8.0 and 21°C with 25 mg crosslinker ($BS^3$) per mg membrane protein for 0.5, 1, 5, 10, 15, and 30 minutes, respectively. In (c), lanes 1 to 6 represent the incubation of microsomes for 30 min at 21°C with 25 mg crosslinker ($BS^3$) per mg membrane protein using pH 5.0, pH 6.0, pH 7.0, pH 8.0, pH 9.0, and pH 10.0, respectively. In (d), lanes 1 to 5 represent the incubation of microsomes for 30 min and pH 8.0 with 25 mg crosslinker ($BS^3$) per mg membrane protein at O°C, 4°C, 10°C, 21°C, and 37°C, respectively. The sizes of molecular mass standards (in kDa) are indicated on the left.*

*Chemical crosslinking analysis of SERCA2 $Ca^{2+}$-ATPase*

Similar results, as shown above for SERCA l, were also achieved for SERCA2. Fig. 5 summarizes the optimization procedure for chemical crosslinking of the slow $Ca^{2+}$-ATPase of the sarcoplasmic reticulum. Using 12.5 to 50 mg crosslinker $BS^3$ per mg membrane protein, a clear decrease in immunolabelling of the SERCA2 monomer and an increase of an apparent tetramer was observed (Fig. 5a). Therefore, subsequent experiments were performed with 25 mg crosslinker per mg protein. A minimum of 10 minutes incubation time resulted in reproducible shifts in the relative electrophoretic mobility of this $Ca^{2+}$-pump isoform (Fig. 5b). Increasing pH-values and increasing temperature caused a decrease in the apparent monomeric protein species (Fig. 5c, d). Overall, using incubation conditions relatively close to the physiologically relevant situation, chemical crosslinking with low ratios between the $BS^3$-probe and native muscle membrane vesicles resulted in the formation of high-molecular-mass SERCA2 complexes.

## Discussion

One of the major aims of basic myology research is to understand structure-function relationships in skeletal muscle proteins. This includes the biochemical analysis of protein-protein interactions and complex formation, since oligomerization might be involved in signal transduction pathways, cooperative kinetics, protein stabilization and protection of individual peptide

subunits against proteolytic degradation. Many key $Ca^{2+}$-regulatory muscle proteins, including voltage-sensors, $Ca^{2+}$-channels, $Ca^{2+}$-binding proteins and $Ca^{2+}$-pumps, seem to exist as supra-molecular complexes [31]. Previous investigations into the oligomeric status of components of the sarcoplasmic reticulum clearly revealed that a 110 kDa muscle membrane protein has a strong tendency to form oligomeric complexes [3, 14, 18, 21]. Since the characterization of the crosslinked product was carried out with protein gels and not immunoblotting, these earlier studies could not unobjectionably determine the identity of this protein, although it was assumed to represent SERCA units [1]. However, even though immunoblotting is much more specific than simple protein gels, it is important that antibodies employed in the identification of crosslinked products are highly specific and do not immunologically cross-react with other components of the vesicular membranes preparation investigated. We therefore used previously well characterized probes, monoclonal antibodies IIH11 and IID8, which exclusively recognize the fast SERCA1 and the slow SERCA2 isoforms, respectively, and no other muscle membrane proteins [34].

Although monomeric SERCA molecules are capable of performing individual steps of the $Ca^{2+}$-ATPase reaction cycle [2, 25] and the topological analysis suggests that a single SERCA copy contains a sufficient number of transmembrane helices to constitute an ion channel [6, 41], freeze-fracture electron microscopy clearly revealed that $Ca^{2+}$-ATPase molecules are represented by 8.5 nm intramembranous particles in the sarcoplasmic reticulum membrane system [26, 27]. The optimized crosslinking data presented in this study confirm the idea that under native conditions SERCA exists as a homo-tetrameric unit. Thus, the central ion-regulatory elements involved in the excitation-contraction-relaxation cycle of skeletal muscle fibres form high-molecular-mass complexes. Besides direct physical interactions between the $a_1$-dihydropyridine receptor and the ryanodine receptor $Ca^{2+}$-release channel [32] and protein clustering of calsequestrin [33], protein-protein interactions also seem to play an essential role in the formation of the physiologically active $Ca^{2+}$-pump units of the sarcoplasmic reticulum. Consequently, the $Ca^{2+}$-mediated regulation of signal transduction during excitation-contraction coupling and muscle relaxation involves supra-molecular membrane complexes of the junctional triad structures and the longitudinal tubules.

The flow chart of the optimization procedure shown in Fig. 6 represents approximately 3 days of experimental work. Since we used a mini-gel and a mini-immunoblotting system in our crosslinking analysis, the initial analytical approach to investigating the oligomeric status of a muscle membrane protein is therefore fast, simple, cheap and uses a minimum of tissue material and reagents necessary to determine the optimum conditions

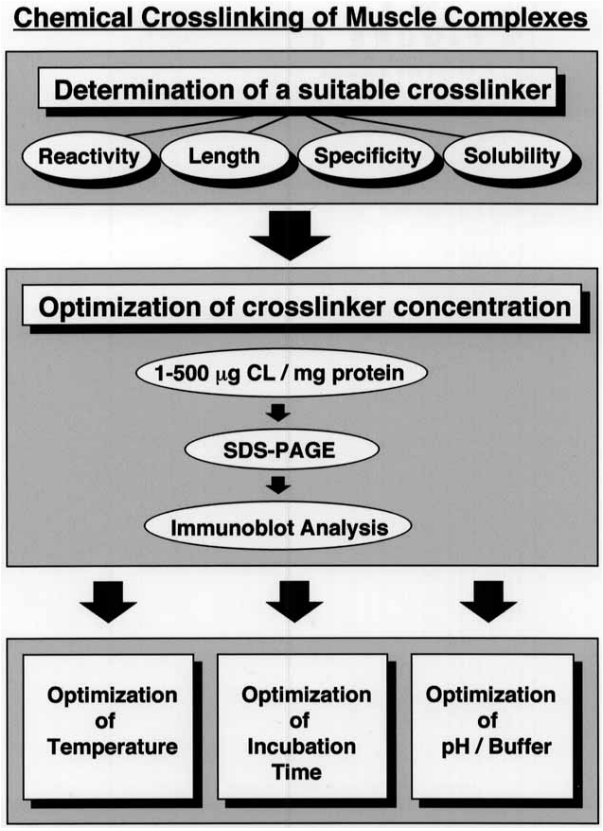


*Figure 6. Flow chart of rapid optimization of chemical crosslinking in the analysis of the oligomeric status of muscle membrane proteins. This study describes a efficient and swift experimental scheme to improve reaction conditions with respect to concentration ratios between crosslinkers and biomembranes, length of incubation time, pH and temperature.*

for subsequent more elaborate and detailed analyses, i.e. comparison between normal and pathological samples or drug/chemical-induced changes in protein-protein interactions. A great variety of homo-bifunctional, heterobifunctional or zero-length crosslinker are now commercially available, as reviewed by Mattson et al. [28]. It is thus important to determine in small-scale pilot experiments which crosslinker is suitable for a particular specification before starting the above outlined optimization procedure. The biological buffer is also important in chemical crosslinking. In order to avoid interference between the buffer system and $BS^3$-induced chemical crosslinking, the buffer has to be free of components containing primary amines. Although the size of chemically crosslinked complexes should not be overestimated [36], as long as the introduction of potential artifacts of random conjugation is prevented [35], crosslinking can be employed as an extremely versatile and reliable analytical method in the determination of protein structures in native biomembranes. Thus, in conjunction with differential co-immunoprecipitation studies, domain binding

experiments, electron microscopical freeze-fracture and/or cryostructure analyses, and co-localization studies using immunofluorescence microscopy, chemical crosslinking can be employed to determine close neighbourhood relationships between muscle membrane proteins.

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