Tobacman, L. S. (1986) *Biophys. J. 49*, 452a.
Tobacman, L. S., & Lee, R. (1987) *J. Biol. Chem. 262*, 4059–4064.
Van Eerd, J. P., & Takahashi, K. (1975) *Biochem. Biophys. Res. Commun. 64*, 122–127.
White, S. P., Cohen, C., & Phillips, G. N., Jr. (1987) *Nature (London) 325*, 826–828.
Wilkinson, J. M. (1978) *Biochem. J. 169*, 229–238.
Wilkinson, J. M., & Grand, R. J. A. (1975) *Biochem. J. 149*, 493–496.
Wilkinson, J. M., & Taylor, R. D. (1986) *J. Mol. Cell. Cardiol. 18*, 291–297.
Wilkinson, J. M., Moir, A. G., & Waterfield, M. D. (1984) *Eur. J. Biochem. 143*, 47–56.

Cross-Linking of Rabbit Skeletal Muscle Troponin with the Photoactive Reagent 4-Maleimidobenzophenone: Identification of Residues in Troponin I That Are Close to Cysteine-98 of Troponin C†

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ABSTRACT: We have used the sulfhydryl-specific, heterobifunctional, photoactivatable cross-linker 4-maleimidobenzophenone (BPMal) to study the interaction of rabbit skeletal muscle troponin C (TnC) and troponin I (TnI). TnC was specifically labeled at Cys-98 by the maleimide moiety of BPMal, and a binary complex was formed with TnI in the presence of Ca^{2+}. Upon photolysis, covalent cross-links were formed between TnC and TnI [Tao, T., Scheiner, C. J., & Lamkin, M. (1986) *Biochemistry 25*, 7633–7639]. The cross-linked heterodimer was digested with cyanogen bromide, pepsin, and chymotrypsin into progressively smaller cross-linked peptides, which were purified by HPLC and then characterized by amino acid analysis and sequencing. We obtained a fraction from the initial CNBr digest that contained the expected peptide CB9 (residues 84–135) of TnC, cross-linked mainly to CN4 (residues 96–116), the "inhibitory region" of TnI. The peptides CN1 and CN3 of TnI were also detected in this fraction, but their molar ratios (compared to CB9) were only about 0.15 each, compared to 0.60 for CN4. Sequence analyses of fractions obtained after peptic and chymotryptic digests of the cross-linked CNBr fraction confirmed that CB9 and CN4 were the major cross-linked species. Quantitative analysis of sequencer results indicated that the residues in TnI that appeared to be most highly cross-linked to Cys-98 of TnC were Arg-108 and Pro-110, and to a lesser extent Arg-103 and Lys-107. These findings are consistent with previous studies on interactions between TnI and TnC and provide, for the first time, direct information on the identities of proximate amino acids in the two proteins.

The interaction of actin and myosin that occurs during muscle contraction is regulated by changes in intracellular Ca^{2+} concentration. In vertebrate striated muscles the dominant regulatory system involves binding of Ca^{2+} to troponin in the thin filaments. Troponin is a complex of three different protein subunits: TnC[1] binds Ca^{2+}, TnI binds to actin and inhibits actin–myosin interaction, and TnT binds to tropomyosin. The amino acid sequences of all three rabbit fast skeletal muscle troponin subunits have been determined (Wilkinson & Grand, 1975; Collins et al., 1973, 1977; Pearlstone et al., 1977), and these proteins have served as the models for extensive structure–function studies carried out in several laboratories [see Leavis and Gergely (1984) for review]. While much remains to be learned about the three-dimensional structure of rabbit skeletal muscle TnI and TnT, analysis of the TnC sequence (Collins et al., 1973) predicted the locations of four Ca^{2+}-binding sites, designated I–IV going from the amino to the carboxyl terminus of the protein. This prediction was confirmed by the more recently available crystal structures of chicken (Sundaralingam et al., 1985) and turkey (Herzberg & James, 1985) TnCs.

Many studies have focused on the mechanism of the transmission of Ca^{2+}-induced structural changes from one thin filament protein to another (Leavis & Gergely, 1984). A key aspect of this problem is the elucidation of interactions among the thin filament proteins, particularly those among the three troponin components. In the case of the binding of TnC to TnI, considerable information has been obtained from studies of the binding of various proteolytic fragments (Grabarek et al., 1981; Syska et al., 1976), from the reactivities of surface

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[1] Abbreviations: CNBr, cyanogen bromide; TFA, trifluoroacetic acid; PTH, phenylthiohydantoin; PTC, phenylthiocarbamyl; HPLC, high-performance liquid chromatography; TnC, troponin C; TnI, troponin I; BPMal, 4-maleimidobenzophenone; BP-TnC, TnC labeled with BPMal; BP-TnC*I, cross-linked heterodimer of TnI and BP-TnC; BP-CB9*CN4, cross-linked CNBr peptide from BP-TnC*I.

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amino acids (Hitchcock, 1981), and from various spectroscopic studies (Leavis & Tao, 1980). There appear to be at least two sites of interaction between TnC and TnI, but little is known about the identities of the particular amino acid residues that make up these sites.

Tao et al. (1985a, 1986) recently demonstrated that BPMal, a thiol group specific, high-yield photochemical cross-linker, shows great promise as a probe of thin filament protein interactions. In particular, they showed that in the presence of Ca^{2+} BP-TnC (labeled at Cys-98) will form binary complexes with either TnI or TnT and that the proteins can be covalently linked together by photo-cross-linking. These same cross-linked heterodimers are also formed after photoactivation of the ternary complex of BP-TnC, TnI, and TnT. Cross-linking yields were not Ca^{2+} dependent. The cross-linking is not due to random collisions between the proteins, since no cross-linking occurs under conditions that dissociate the complex. The purpose of the present study is to characterize the nature of these cross-links in order to identify amino acid residues in TnI that lie close to Cys-98 of TnC. Labeling of TnC with BP-Mal does not appear to alter its functional properties (Tao et al., 1986), so cross-links formed with TnI are likely to accurately reflect interactions that occur between the native proteins.

Experimental Procedures

Materials. All reagents were of the highest grade commercially available. Sequencer reagents were from Applied Biosystems. PTH-amino acid standards were from either Pierce or Applied Biosystems. Amino acid standards, PITC (for nonsequencer use), and constant-boiling HCl were obtained from Pierce. HPLC-grade water and TFA were obtained from Fisher. HPLC-grade acetonitrile was obtained from Burdick and Jackson. All other chemicals were obtained from Fisher.

Amino Acid Compositions and Sequence Analysis. Phenylthiocarbamylated acid hydrolysates of peptides were prepared with the Waters PICO-TAG work station. PTC-amino acids were analyzed by reverse-phase HPLC (Heinrikson et al., 1984; Bidlingmeyer et al., 1984; Cohen et al., 1986) on a Waters PICO-TAG column, with the gradient elution system recommended by the manufacturer. Amino acid sequences were determined with an Applied Biosystems Model 470A gas-phase protein sequencer as described by Hewick et. al. (1981). PTH-amino acids obtained from the sequencere were also analyzed by reverse-phase HPLC, with a Waters Nova-Pak column and the gradient elution system described in Waters Associates Applications Brief M3500. The recovery of PTH-amino acids at each cycle was measured quantitatively. PTH-Ser and PTH-Thr were usually obtained in low yields and sometimes not detectable at all; however, they could always be identified by the appearance of breakdown products, which absorbed at 313 nm. PTH-Arg and PTH-His were also often recovered in low yields. A Waters HPLC system including two M510 pumps, a M721 system controller, a WISP 710A autoinjector, a temperature control module, a M440 dual-channel absorbance detector, and a M730 integrative recorder was used for both PTH- (sequence) and PTC- (composition) amino acid analyses. The detector was set at 254 nm to measure PTC-amino acids and the sum of 254 nm (to measure quantitatively PTH-amino acids) and 313 nm (for qualitative detection of breakdown products of PTH-Ser and PTH-Thr).

Preparative HPLC. All peptide mixtures were separated by reverse-phase HPLC on a 4.6 mm × 25 cm Vydac 218TP54 column. Two Waters M510 pumps, a Waters M680 controller, a Waters M480 variable-wavelength absorbance detector, a Linear dual-channel recorder, and a Glenco SV-3 injector were used as our HPLC system. Solvent A was 0.1% TFA in acetonitrile–water (5:95 v/v), and solvent B was 0.1% TFA in acetonitrile–water (95:5 v/v). All separations were carried out in a linear gradient from 0% B to 40% B at a flow rate of 1 mL/min. The eluent was monitored at 220 nm.

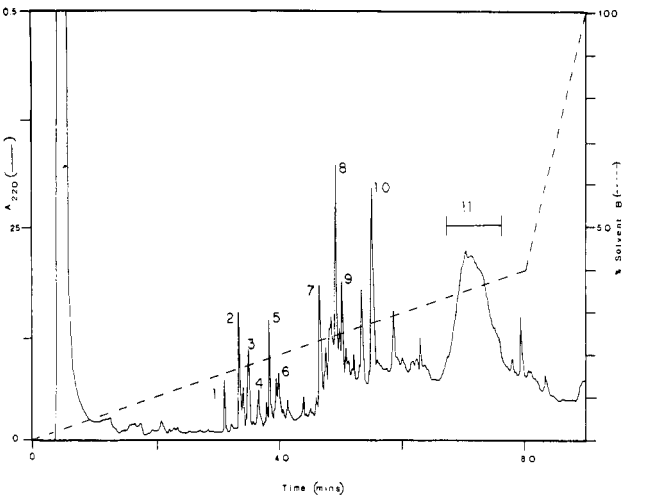

FIGURE 1: HPLC of a CNBr digest of 18 nmol of BP-TnC*I. CN designates CNBr peptides of TnI (Wilkinson & Grand, 1978), and CB designates CNBr peptides of TnC (Collins et al., 1977). The identities of the numbered fractions are as follows: 1, CN6; 2, CN5; 3, CN5; 4, CN7; 5, CB5; 6, CB5; 7, CB4; 8, CN2; 9, CN2; 10, CB7; 11, BP-CB9*CN4. Some peptides eluted as two peaks, due to the homoserine to homoserine lactone conversion.

Preparation of Cross-Linked Peptides. The cross-linked heterodimer BP-TnC*I was prepared from the binary complex of rabbit skeletal muscle TnI and BP-TnC as described by Tao et al. (1986). BP-TnC*I (73.5 nmol) was dissolved in 300 μL of 70% formic acid. To this was added 7.40 mg of CNBr dissolved in 70% formic acid. The reaction occurred at room temperature with constant stirring for 19.5 h. The reaction mixture was diluted to 3 mL with water and dried under nitrogen and then dissolved in 1 mL of water and dried again under nitrogen. The sample was then dissolved in 300 μL of 70% formic acid and applied directly to the HPLC column. The cross-linked fraction (20 nmol) obtained from the CNBr digest was dissolved in 200 μL of 5% formic acid. To this was added 30 μg of pepsin (Worthington, 25 000 units/mg) dissolved in 5% formic acid. The digest solution was stirred for 2.5 h at 25 °C and then applied directly to HPLC. The cross-linked fraction (8 nmol) obtained from the peptic digest was dissolved in 300 μL of digest buffer (0.1 M ammonium bicarbonate, 0.1 mM calcium chloride, pH 8.1). To this was added 20 μg of α-chymotrypsin (Worthington, 61 units/mg) freshly dissolved in digest buffer. Digestion occurred for 19 h at 37 °C. The digest solution was applied directly to HPLC.

Results

HPLC of the CNBr digest of BP-TnC*I (Figure 1) yielded various fractions that were identified by amino acid analysis (not shown) as CNBr peptides from both TnC (Collins et al., 1977) and TnI (Wilkinson & Grand, 1975). In addition, we obtained an unusual, late-eluting, broad fraction (Figure 1, fraction 11). Amino acid analysis indicated that fraction 11 contained CB9 (residues 84–135 of TnC, which includes the single Cys residue at position 98) and CN4 (residues 96–116 of TnI) as its major componets. Control HPLC runs (not shown) on CNBr digests of un-cross-linked TnI and TnC were carried out to comfirm that CN4 and CB9 were not simply

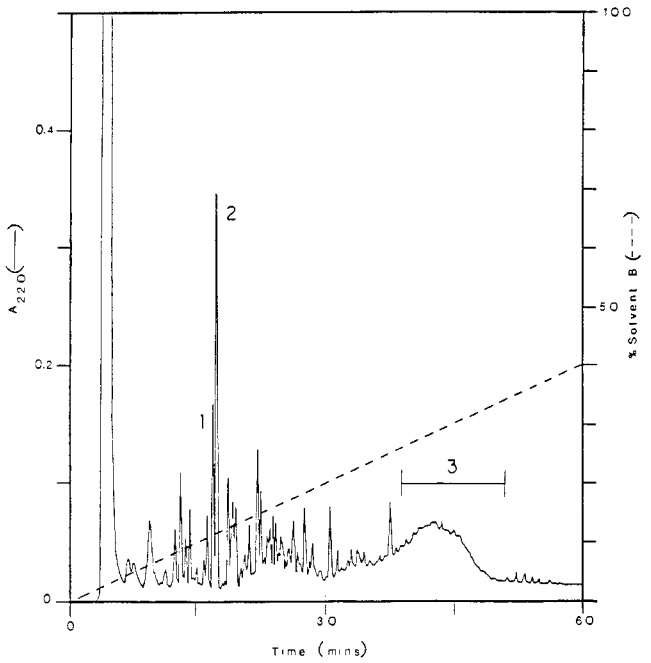

FIGURE 2: HPLC of the peptic digest of 20 nmol of BP-CB9*CN4 (fraction 11 from Figure 1). Fractions 1 and 2 were identidied as TnC (109–113) and TnC (109–114), respectively, and fraction 3 was the cross-linked material.

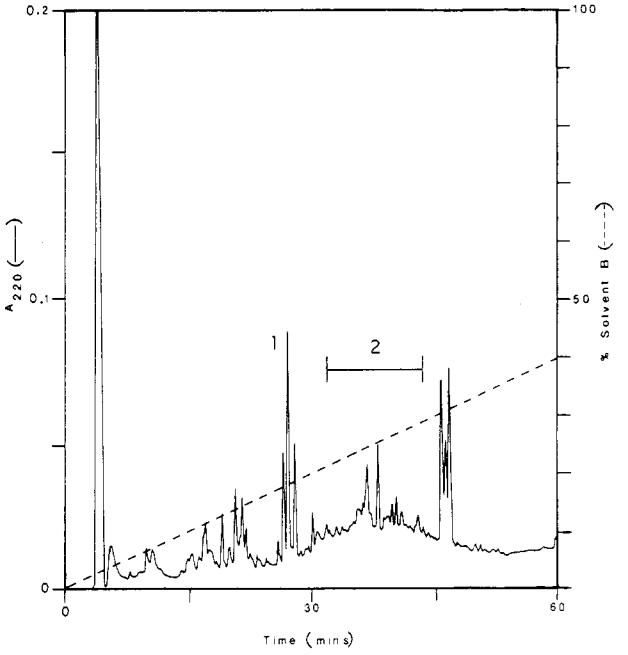

FIGURE 3: HPLC of the chymotryptic digest of 8 nmol of cross-linked peptic peptides (fraction 3 from Figure 2). Fraction 1 was identified as TnI (101–106), and fraction 2 was the cross-linked material.

coeluting in fraction 11. We found that free CN4 elutes just prior to CN2, well ahead of fraction 11. Free CB9 elutes as a heterogeneous peak, slightly later than fraction 11. Although free CB9 could partially coelute with fraction 11, this is very unlikely since the starting material for CNBr digestion contained only dimeric, cross-linked BP-TNC*I. Additional evidence against coelution of CN4 and CB9 is that further digests (see below) of fraction 11 yielded fractions that continued to contain peptide fragments from both CB9 and CN4.

Sequence analysis of fraction 11 showed that the cross-linked peptide BP-CB9*CN4 was its major component. We identified four simultaneously appearing sequences: CB9, CN4, CN1 (residues 22–57 of TnI), and CN3 (residues 58–81 of TnI). From quantitative analysis of recovered PTH-amino acids, we estimate that approximately 60% of the CB9 is cross-linked to CN4, while the remainder is cross-linked to CN1 (15%), CN3 (15%), and minor peptides (total 10%) that could not be identified. It should be kept in mind, however, that it was very difficult to accurately quantify the relative amounts of each peptide, due to complexities caused by variable yields, multiple sequences, and carryover of PTH-amino acids from one cycle to the next. The sequencing of CB9 appeared to proceed normally, except for the absence of an identifiable PTH-amino acid corresponding to cross-linked Cys-98. All the expected PTH-amino acids of CN4 were also accounted for, but those of Arg-103, Lys-107, Arg-108, and Pro-110 were recovered in lower yields (compared to a control sequence analysis on un-cross-linked CN4), suggesting that these residues may be partially cross-linked to Cys-98 of TnC. This identification was possible because CB9 and CN4 yielded different PTH-amino acids through most of the sequence analysis. The only exception was cycle 17, in which CB9 and CN4 both yielded PTH-Arg. Because of this, it was not possible to attribute apparent low yields of PTH-Arg in cycles 17 and 18 to cross-linking of Arg-112 and Arg-113 of TnI to Cys-98 of TnC. Contributions from CN1 and CN3 did not significantly interfere with these identifications.

To more precisely identify the major cross-linked residues in TnI, the CNBr fraction 11 was further broken down with pepsin. After HPLC of the peptic digest, a late-eluting, broad fraction of cross-linked material was again obtained (Figure 2, fraction 3). Amino acid analysis of this heterogeneous fraction did not permit us to identify the peptides present. After sequence analysis, however, two TnC peptides (residues 95–99 and 96–99, arising from partial cleavage at Leu-95) and a TnI peptide (residues 100–116) were clearly identified. This fraction also gave a high background of several PTH-amino acids at each cycle, precluding the identification of low-yield PTH amino acids derived from individual cross-linked residues in TnI. The total molar ratio of CN4-derived to CB9-derived peptides, as estimated from quantitative analysis of the three identifiable sequences, was 0.30, about half the ratio of CN4 to CB9 in the intact CNBr fraction. Because of steric hindrance from the large number of non-specific cross-links between CN4 and the bulky benzophenone moiety of BPMal, pepsin undoubtedly cleaved CB9 much more quantitatively and cleanly than it did CN4. After peptic cleavage therefore, the number of TnI-derived peptides, relative to those from TnC, increased substantially. It should also be kept in mind that CN4 represents only about 60% of the TnI peptides that are cross-linked to CB9. Most of the TnI peptides are present in low yield and during sequence analysis would show up as a relatively high background of several PTH-amino acids, as we observed. One might also expect that the removal by pepsin of non-cross-linked regions of CN4 would eliminate a significant fraction of the PTH-amino acids that we detect by sequence analysis. This should not, however, be a major factor in decreasing the ratio of TnI to TnC sequences, since the total amount of all cross-linked residues in TnI-derived peptides can be no greater than the amount of Cys-98, the single, quantitatively cross-linked residue of TnC.

The cross-linked peptic fraction was further digested with chymotrypsin, and after HPLC a very broad fraction of cross-linked material (Figure 3, fraction 2) was obtained. Amino acid analysis of the this fraction was once again useless for identifying the cross-linked peptides. Sequence analysis, however, showed that significant further degradation of TnI-derived peptides had taken place, while no further breakdown of TnC-derived peptides had occurred. We iden-

tified three sequences: Leu-Ala-Glu-X-Phe (residues 95–99 of TnC, where X is the cross-linked Cys-98), Ala-Glu-X-Phe (residues 96–99 of TnC), and Lys-Arg-Pro-Pro-Leu (residues 107–111 of TnI). On the basis of sequencer yields, the molar ratio of TnI-derived to TnC-derived chymotryptic peptides was approximately 0.25, close to the value obtained from the peptic cross-linked fraction. This indicates that chymotryptic removal of residues 100–106 and 112–116 of TnI from the peptic cross-linked fraction did not result in a significant increase in heterogeneity. Indeed, from the reduced yields of their PTH-amino acids, we were able to identify Arg-108 and Pro-110 of TnI as the predominant cross-linked residues in this fraction.

Discussion

The dual functionality of BPMal allowed us to specifically label Cys-98, the sole Cys residue of TnC, through the maleimide moiety. The labeled TnC was allowed to form a complex with TnI in the presence of Ca^{2+}, and photoactivation of the benzophenone group gave rise to a triplet diradical that nonspecifically formed covalent cross-links with nearby carbon atoms. After purification of the dimeric BP-TnC*I, we prepared a series of progressively smaller cross-linked peptides, which were characterized by amino acid analysis and sequencing. Amino acid analysis of the initial digest permitted us to identify a CNBr fraction that contained the cross-linked peptides CB9 (from TnC) and CN4 (from TnI). Sequence analysis of this fraction confirmed the identification and extended the characterization of the cross-linked material. The sequences of CN1 and CN3 of TnI were also detected in this fraction, but the molar ratios (compared to CB9) were only about 0.15 each, compared to 0.60 for CN4. In subsequent peptic and chymotryptic digests of the cross-linked CNBr fraction, amino acid analysis was not useful for identification purposes. Sequence analysis, however, unambiguously identified cross-linked peptides derived from CB9 and CN4, despite the heterogeneity of the cross-linked fractions. Peptides derived from CN1 and CN3 were not detected in cross-linked fractions obtained from the peptic and chymotryptic digests.

We found that the residues in TnI that appeared to be most highly cross-linked to Cys-98 of TnC were Arg-108 and Pro-110, and to a lesser extent Arg-103 and Lys-107. These results are consistent with previous findings (Dalgarno et al., 1982; Cachia et al., 1983) that residues 89–100 of TnC interact with residues 96–116 of TnI in the troponin complex. Changes in the environments of residues in CN4, and synthetic analogues of CN4, upon binding to TnC and actin, were investigated in the proton NMR studies of Grand et al. (1982). They found that the environments of all five Arg (spanning residues 103–115) in CN4 were perturbed upon binding to actin, whereas all of the Lys, Leu, and Phe (total of eight, spanning residues 98–111) were perturbed upon binding to TnC. Our results indicate that the highest concentration of cross-links was in residues 107–111 of TnI, particularly Arg-108 and Pro-110. It should be kept in mind that we are looking at residues in whole TnI that are close enough to form cross-links with a single residue (Cys-98) in TnC. Grand et al. (1982) were measuring perturbations in small TnI-derived peptides that were probably caused by interactions with several residues in TnC and actin. When one takes into account the different phenomena being examined, the results of these two studies agree remarkably well.

Hydropathy analysis (Kyte & Doolittle, 1982) of the TnI sequence (not shown) indicates that CN4 (residues 96–116) is in a hydrophilic region, probably forming part of the external surface of the TnI molecule, as would be expected for interaction with TnC. A previous analysis (Wilkinson & Grand, 1975) of the possible secondary structure of TnI predicted that within this region residues 109–112 may form a β-turn. Judging from the results of the present study, several residues in the CN4 region, particularly Arg-108 and Pro-110, of TnI lie within 10 Å (the mean molecular distance between attachment sites for BPMal) of Cys-98 of TnC.

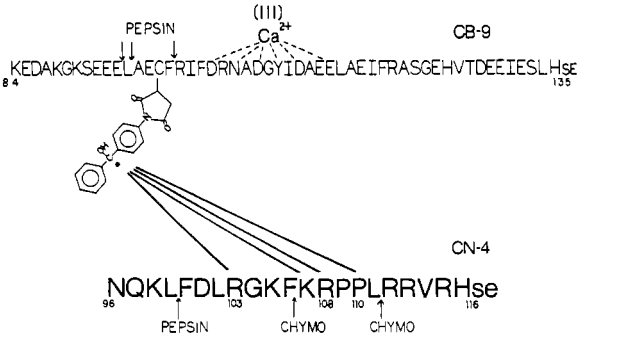

FIGURE 4: Amino acid sequences of BP-CB9*CN4 in the single-letter code. In CB9, Ca^{2+} is shown with its coordinating ligands in calcium binding site III. The cleavage sites of the subsequent peptic and chymotryptic digests are indicated. The BPMal is shown attached to Cys-98 as a radical, and several connecting lines to CN4 are drawn to indicate that several attachment sites are possible. Amino acids that were implicated as major cross-linking sites are Arg-103, Lys-107, Arg-108, and Pro-110.

Our results provide more definitive information than has been obtained from previous cross-linking studies, which have been carried out on the troponin subunits. Hitchcock (1975) and Sutoh (1980) used cross-linkers that attach to multiple Lys residues in these proteins, yielding extremely complex mixtures of cross-linked species. Sulfhydryl-specific cross-linkers have generally proven to be more useful, since they permit the study of protein interactions at a limited number of specific sites. In one study (Chong et al., 1981) TnC was labeled (presumably at Cys-98) with *N*-[(4-azidobenzoyl)[2-^{3}H]glycyl]-*S*-(2-pyridylthio)cysteine and then cross-linked to TnI. The identities of the cross-linked residues were not determined, but a later publication from the same laboratory (Cachia et al., 1983) cited unpublished results that showed that Cys-98 of TnC was cross-linked to CN4 of TnI. Dobrovl'sky et al. (1984) reacted whole troponin with 1,3-difluoro-4,6-dinitrobenzene, forming a cross-linked TnC–TnI complex. They isolated an apparent cross-linked CNBr peptide whose amino acid composition resembled the sum of CB9 of TnC and CN7 (residues 122–134) of TnI. On the basis of the presumption (not directly confirmed) that their reagent had reacted exclusively with Cys residues, they concluded that Cys-98 of TnC had formed a cross-link with Cys-133 of TnI. The formation of this Cross-link was promoted by the absence of Ca^{2+}. In contrast, fluorescence energy transfer measurements (Tao et al., 1985b; Wang & Cheung, 1986) indicate that the minimum distance between these Cys residues in the binary TnC–TnI complex is 34 Å, much too great for signifcant cross-linking to occur.

Our cross-linked peptide fractions always eluted from HPLC as broad peaks. This is probably due to their heterogeneous populations, arising from different linkage sites on TnI (see Figure 4). The benzophenone radical formed cross-links with several amino acid residues in TnI, and each amino acid side chain probably had several attachment sites. Furthermore, the formation of new carbon–carbon bonds from carbon–hydrogen bonds significantly increases the chirality of the cross-linked amino acids, resulting in an even more diverse population. The cross-linked fractions appeared to become broader as they were further digested into smaller peptides,

reflecting the increased contribution of molecular diversity in the cross-linked TnI residues. The progressive removal of un-cross-linked amino acid residues left behind an increasingly heterogeneous mixture of small, cross-linked peptides whose variability could no longer be masked by the constant portions. This was especially evident when peptic digestion of the cross-linked CNBr fraction removed most of the large (52 residues) CB9 of TnC.

Previous binding studies on proteolytic fragments of rabbit skeletal muscle TnC have shown that three regions (residues 50–60, 89–100, and 126–136) can bind to whole TnI. One of these (residues 126–136) binds independently of Ca^{2+}, while the other two bind only when TnC is saturated with Ca^{2+} (Grabarek et al., 1981). All three fragments are located in regions of TnC that form helices that contain acidic amino acids clustered along one face (Grabarek et al., 1981; Herzberg & James, 1985; Sundaralingam et al., 1985). Similar binding studies on rabbit skeletal TnI fragments have shown that the basic peptides "CF2" (residues 1–47) and CN4 (residues 96–116) bind to whole TnC (Syska et al., 1976). It is likely that acidic surface regions of TnC interact with basic surface regions of TnI in an electrostatic manner.

In the present study, we have more precisely defined the interaction of TnI and TnC by showing that Cys-98 (included within CB9 and the binding fragment 89–100) lies within 10 Å of several residues (Arg-103, Lys-107, Arg-108, Pro-110) that are included in CN4. This finding may shed additional light on the manner in which TnC and TnI act to regulate the interaction of actin and myosin. CN4 is a remarkable peptide that retains 40–75% of the inhibitory activity of whole TnI and also binds to actin (Syska et al., 1976; Leavis & Gergely, 1984; Zot & Potter, 1987). Residues 89–100 of TnC undergo a coil to helix transformation upon addition of Ca^{2+} (Nagy et al., 1978; Leavis et al., 1978). This would explain the Ca^{2+} sensitivity of the binding of TnC fragment 89–100 to TnI (Syska et al., 1976; Grabarek et al., 1981). Binding of Ca^{2+} to sites I and II in the amino-terminal half of TnC is believed to trigger activation of the thin filament [see Leavis and Gergely (1984) and Zot and Potter (1987) for reviews]. Recent kinetic studies (Grabarek et al., 1986) have shown that the binding of Ca^{2+} to these sites also perturbs the structure of the Cys-98 region (near Ca^{2+} binding site III), possibly facilitating the interaction of this residue with CN4 (the TnI inhibitory region). Binding of CN4 to TnC fragment 89–100 probably involves hydrophobic as well as electrostatic interactions, and CN4 may interact with actin in a similar fashion [see discussion by Cachia et al. (1983)]. In muscle, the CN4 region of TnI interacts strongly with actin in the absence of Ca^{2+}; then, in the presence of Ca^{2+} CN4 would bind more strongly to TnC, and there would be a weakening of the inhibitory activity of TnI.

In conclusion, we have demonstrated the utility of BPMal for characterizing sites of interaction between TnC and TnI. The quantitative and specific labeling of TnC at Cys-98, combined with the high-efficiency photo-cross-linking of nearby amino acid residues on TnI, allowed us to characterize cross-links that may not form in significant amounts with other cross-linking agents. The correlation of biochemical characterization of cross-linked materials (Tao et al., 1986) and identification of cross-linked amino acids provides a powerful procedure for elucidating the complex protein interactions that take place during muscle contraction and relaxation.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL AVAILABLE

Tables I–IV containing amino acid compositions and sequence data for cross-linked fractions (2 pages). Ordering information is given on any current masthead page.

Registry No. Cys, 52-90-4; Arg, 74-79-3; Pro, 147-85-3; Lys, 56-87-1.

REFERENCES

Bidlingmeyer, B. A., Cohen, S. A., & Tarvin, T. L. (1984) *J. Chromatogr. 336*, 93–104.

Cachia, P. J., Sykes, B. D., & Hodges, R. S. (1983) *Biochemistry 22*, 4145–4152.

Chong, P. C. S., & Hodges, R. S. (1981) *J. Biol. Chem. 256*, 5071–5076.

Cohen, S. A., Bidlingmeyer, B. A., & Tarvin, T. L. (1986) *Nature (London) 320*, 769–770.

Collins, J. H., Potter, J. D., Horn, M. J., Wilshire, G., & Jackman, N. (1973) *FEBS 36*, 268–272.

Collins, J. H., Greaser, M. L., Potter, J. D., & Horn, M. J. (1977) *J. Biol. Chem. 252*, 6357–6362.

Dalgarno, D. C., Grand, R. J. A., Levine, B. A., Moir, A. J. G., Scott, G. M. M., & Perry, S. V. (1982) *FEBS Lett. 150*, 54–58.

Dobrovl'sky, A. B., Gusev, N. B., & Friedrich, P. (1984) *Biochim. Biophys. Acta 789*, 144–151.

Grabarek, Z., Drabikowski, W., Leavis, P. C., Rosenfeld, S. S., & Gergely, J. (1981) *J. Biol. Chem. 256*, 13121–13127.

Grabarek, Z., Leavis, P., & Gergely, J. (1986) *J. Biol. Chem. 261*, 608–613.

Grand, R. J. A., Levine, B. A., & Perry, S. V. (1982) *Biochem. J. 203*, 61–68.

Heinrikson, R. L., & Meredith, S. C. (1984) *Anal. Biochem. 136*, 65–74.

Herzberg, O., & James, M. (1985) *Nature (London) 313*, 653–659.

Hewick, R. M., Hunkapillar, M. W., Hood, L. E., & Dreyer, W. J. (1981) *J. Biol. Chem. 256*, 7990–7997.

Hitchcock, S. E. (1975) *Biochemistry 14*, 5162–5167.

Hitchcock, S. E. (1981) *J. Mol. Biol. 147*, 153–173.

Kyte, J., & Doolittle, R. F. (1982) *J. Mol. Biol. 157*, 105–132.

Leavis, P. C., & Tao, T. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 724.

Leavis, P. C., & Gergely, J. (1984) *CRC Crit. Rev. Biochem. 16*, 235–305.

Leavis, P. C., Rosenfeld, S. S., Gergely, J., Grabarek, Z., & Drabikowski, W. (1978) *J. Biol. Chem. 254*, 5452–5459.

Nagy, B., Potter, J. D., & Gergely, J. (1978) *J. Biol. Chem. 253*, 5971–5974.

Pearlstone, J. R., Johnson, P., Carpenter, M. R., & Smillie, L. B. (1977) *J. Biol. Chem. 252*, 983–989.

Potter, J. D., & Gergely, J. (1975) *J. Biol. Chem. 250*, 4628–4633.

Sundaralingam, M., Bergstrom, R., Strasburg, G., Rao, S. T., Roychowdhury, P., Greaser, M., & Wang, B. C. (1985) *Science (Washington, D.C.) 227*, 945–948.

Sutoh, K. (1980) *Biochemistry 19*, 1977–1983.

Syska, H., Wilkinson, J. M., Grand, R. J. A., & Perry, S. V. (1976) *Biochem. J. 153*, 375–387.

Tao, T., Lamkin, M., & Scheiner, C. J. (1985a) *Arch. Biochem. Biophys. 240*, 627–634.

Tao, T., Strasburg, G., Gowell, E., & Leavis, P. C. (1985b) *Biophys. J. 47*, 509a.

Tao, T., Scheiner, C. J., & Lamkin, M. (1986) *Biochemistry 25*, 7633–7639.

Wang, C.-K., & Cheung, H. C. (1986) *J. Mol. Biol. 190*, 509–521.
Wilkinson, J. M., & Grand, R. J. A. (1975) *Biochem. J. 149*, 493–496.
Wilkinson, J. M., & Grand, R. J. A. (1978) *Nature (London) 271*, 31–35.
Zot, A. S., & Potter, J. D. (1987) *Annu. Rev. Biophys. Biophys. Chem. 16*, 535–559.

Calcium-Activated, Phospholipid-Dependent Protein Kinases from Rat Liver: Subcellular Distribution, Purification, and Characterization of Multiple Forms†

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ABSTRACT: Three forms of Ca^{2+}- and phospholipid-dependent protein kinase (protein kinase C) were extensively purified from rat liver homogenate. Subcellular fractionation analysis indicated that the majority (~85%) of the activity was associated with particulate fractions of the liver. Among these, the microsomal and nuclear fractions accounted for ~63% and ~10% of total activity. The remaining 15% of protein kinase C was recovered in the soluble fraction following differential centrifugation. It was also found that most of the membrane-associated protein kinase C was latent, with 4–6-fold stimulation with detergents such as 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate, octyl β-glucoside, or Triton X-100. The activity of both the bound form and the soluble enzyme was enhanced by the addition of Ca^{2+} and phosphatidylserine, when histone H_1 was used as substrate. The bound protein kinase C activity was dissociated by homogenization of liver in buffer containing ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, ethylenediaminetetraacetic acid, and various proteolytic inhibitors, and the solubilized extract was used to purify multiple forms of the enzyme. The purification procedure sequentially utilized $(NH_4)_2SO_4$ fractionation, ion-exchange chromatography on DEAE-cellulose, gel permeation chromatography on Fractogel TSK HW-55 (F), ion-exchange chromatography on hydroxylapatite, gel permeation chromatography on Ultrogel AcA34, and affinity chromatography on polyacrylamide-immobilized phosphatidylserine. On hydroxylapatite columns, protein kinase C activity was resolved into three isoenzymic forms designated C-I, C-II, and C-III. The molecular weights of the three isoenzymic forms were in the range of 208 000–225 000 as shown by chromatography on calibrated Ultrogel AcA34 columns and sucrose density gradient centrifugation. Furthermore, all three isoenzymes demonstrated a single peak with a sedimentation coefficient ($s_{20,w}$) in the range of 9.0–9.2. However, with polyacrylamide gel electrophoresis, all the forms showed a single protein component with average molecular weight of 64K, suggesting that the native isoenzymes may be composed by subunits. Finally, all three isoenzymes exhibited nearly identical enzymatic properties.

The unique feature of protein kinase C[1] is its dependence on phospholipids (especially phosphatidylserine) as a cofactor (Takai et al., 1979; Kaibuchi et al., 1981). This characteristic as well as the enzyme's requirement for Ca^{2+} (Inoue et al., 1977; Takai et al., 1979; Kaibuchi et al., 1981), its ubiquitous presence in eukaryotes (Kuo et al., 1980; Minakuchi et al., 1981), and its reported dual membrane and cytosolic distribution in cells (Kraft & Anderson, 1983a; Farrar et al., 1985; Wooten & Wrenn, 1985; Tanabe et al., 1985; Naor et al., 1985) suggests a potential role for protein kinase C in membrane events during cell secretion (Nishizuka et al., 1984; Nishizuka, 1984; Berridge & Irvine, 1984). With the recent information that protein kinase C acts as the intracellular receptor site for agents such as phorbol esters (Costagna et al., 1982; Ashendel, 1985), which have been shown to stimulate protein and steroid secretion (Drust & Martin, 1984; Berridge & Irvine, 1984; Gunther, 1981; Putney et al., 1984; Negro-Vilar & Lapetina, 1985; Kojima et al., 1983; Lin, 1985; Kawai & Clark, 1985; Brunswig et al., 1986), this view of protein kinase C as a mediator of intracellular secretory events is strengthened.

To test the interaction between protein kinase C and cellular endomembranes directly, several requirements must be met. First, one must have in possession a purified form of protein kinase C that is totally free of contaminating cytosolic and

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[1] Abbreviations: CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; NP-40, Nonidet P-40 [(octylphenoxy)poly(ethoxyethanol)]; Zwittergent 3-10, zwitterionic (Zwittergent) detergent 3-10; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; protein kinase C, calcium-activated and phospholipid-dependent protein kinase; protein kinase A, adenosine cyclic 3′,5′-monophosphate dependent protein kinase; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); MOPS, 3-(*N*-morpholino)propanesulfonic acid; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone [1-chloro-3-(tosylamido)-7-amino-L-2-heptanone]; TPCK, N^{α}-tosyl-L-phenylalanine chloromethyl ketone [L-1-(tosylamido)-2-phenylethyl chloromethyl ketone]; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; cAMP, adenosine cyclic 3′,5′-phosphate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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