Fernandez-Belda, F., Kurzmack, M., & Inesi, G. (1984) *J. Biol. Chem. 259*, 9687–9698.
Froehlich, J. P., & Eller, P. F. (1985) *Biochemistry 24*, 126–136.
Guillain, F., Champeil, P., Lacapere, J.-J., & Gingold, M. P. (1981) *J. Biol. Chem. 256*, 6140–6147.
Guillain, F., Gingold, M. P., & Champeil, P. (1982) *J. Biol. Chem. 257*, 7366–7371.
Guillain, F., Champeil, P., & Boyer, P. D. (1984) *Biochemistry 23*, 4754–4761.
Hasselbach, N., & Koenig, V. (1980) *Z. Naturforsch. C: Biosci. 35C*, 1012–1018.
Ikemoto, N. (1975) *J. Biol. Chem. 250*, 7219–7224.
Inesi, G., Kurzmack, M., Kosk-Kosicka, D., Lewis, D., Scofano, H., & Guimaraes-Motta, H. (1982) *Z. Naturforsch. C: Biosci. 37C*, 685–691.
Makinose, M. (1973) *FEBS Lett. 37*, 140–143.
McIntosh, D. B., & Boyer, P. D. (1983) *Biochemistry 22*, 2867–2875.
McIntosh, D. B., & Davidson, G. A. (1984) *Biochemistry 23*, 1959–1965.
Meissner, G., Conner, G. E., & Fleischer, S. (1973) *Biochim. Biophys. Acta 298*, 246–269.
Nakamoto, R. K., & Inesi, G. (1984) *J. Biol. Chem. 259*, 2961–2970.
Nakamura, J. (1983) *Biochim. Biophys. Acta 723*, 182–190.
Nakamura, Y., & Tonomura, Y. (1982) *J. Biochem. (Tokyo) 91*, 449–461.
Pick, U., & Racker, E. (1979) *Biochemistry 18*, 108–113.
Pickart, C. M., & Jencks, W. P. (1982) *J. Biol. Chem. 257*, 5319–5322.
Scofano, H. M., Vieyra, A., & de Meis, L. (1979) *J. Biol. Chem. 254*, 10227–10231.
Shigekawa, M., & Dougherty, J. P. (1978) *J. Biol. Chem. 253*, 1458–1464.
Shigekawa, M., & Akowitz, A. A. (1979) *J. Biol. Chem. 254*, 4726–4730.
Shigewaka, M., & Kanazawa, T. (1982) *J. Biol. Chem. 257*, 7657–7665.
Shigewaka, M., Wakabayashi, S., & Nakamura, H. (1983) *J. Biol. Chem. 258*, 14157–14161.
Takisawa, H., & Tonomura, Y. (1979) *J. Biochem. (Tokyo) 86*, 425–441.
Takisawa, H., & Makinose, M. (1983) *J. Biol. Chem. 258*, 2986–2992.
Taylor, J. S., & Hattan, D. (1979) *J. Biol. Chem. 254*, 4402–4407.
Watanabe, T., Lewis, D., Nakamoto, R., Kurzmack, M., Fronticelli, C., & Inesi, G. (1981) *Biochemistry 20*, 6617–6625.
Yamada, S., & Ikemoto, N. (1980) *J. Biol. Chem 255*, 3108–3119.
Yamaguchi, M., & Kanazawa, T. (1984) *J. Biol. Chem. 259*, 9526–9531.
Yamaguchi, M., & Kanazawa, T. (1985) *J. Biol. Chem. 260*, 4896–4900.

Site-Specific Photo-Cross-Linking Studies on Interactions between Troponin and Tropomyosin and between Subunits of Troponin†

Terence Tao,* Cathy J. Scheiner, and Mark Lamkin
Department of Muscle Research, Boston Biomedical Research Institute, Boston, Massachusetts 02114, and Department of Neurology, Harvard Medical School, Boston, Massachusetts 02115

Received April 9, 1986; Revised Manuscript Received July 31, 1986

ABSTRACT: We have used the sulfhydryl-specific heterobifunctional photo-cross-linker 4-maleimidobenzophenone (BP-Mal) to study the interactions of rabbit skeletal tropomyosin with troponin and of the troponin subunits with each other. We found that α,α-tropomyosin specifically labeled at Cys-190 with BP-Mal photo-cross-links with all three subunits of troponin with decreasing cross-linking yields in the order of troponin T, troponin I, and troponin C. There was no apparent Ca^{2+} dependence in the cross-linking yields. In separate experiments, we found that troponin C labeled specifically at Cys-98 with BP-Mal photo-cross-links to both troponin I and troponin T in the two binary complexes, as well as in the ternary complex. Again, no Ca^{2+}-dependent changes in the cross-linking yields were detectable. These results are in general agreement with the picture that troponin I and troponin T are in close contact with troponin C near its Cys-98 and that all three troponin subunits are in the proximity of Cys-190 of tropomyosin.

Although it is well established that the contraction of mammalian skeletal muscle is regulated by calcium ions (Ebashi et al., 1969), the molecular mechanism of this process is still not completely understood. It is known that the regulatory proteins troponin (Tn)[1] and tropomyosin (Tm) in the thin filament are involved and that inhibition of actomyosin ATPase activity is reversed when Ca^{2+} is bound to the TnC component of Tn. One model proposes that the binding of

† Supported by a grant from the National Institutes of Health (AM21673). Presented in preliminary form at the 28th Meeting of the Biophysical Society (Tao et al., 1984) and at the Biophysical Discussions, Nov 1985 (Tao et al., 1986).

* Address correspondence to this author at the Department of Muscle Research, Boston Biomedical Research Institute, 20 Staniford St., Boston, MA 02114.

[1] Abbreviations: Tn and Tm, rabbit skeletal troponin and tropomyosin, respectively; TnC, TnI, and TnT, Ca^{2+} binding, inhibitory, and Tm binding subunits of Tn, respectively; S1, chymotryptic myosin subfragment 1; BP-Mal, 4-maleimidobenzophenone; AGTC, *N*-[(4-azidobenzoyl)glycyl]-*S*-(2-thiopyridyl)cysteine; BP-Tm, $\alpha\alpha$Tm labeled at Cys-190 with BP-Mal; BP-TnC, TnC labeled at Cys-98 with BP-Mal; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; GdmCl, guanidinium chloride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

0006-2960/86/0425-7633$01.50/0

Ca^{2+} to TnC affects first the conformation of TnC and then the interactions of the Tn subunits with each other, of Tn and Tm with actin, and, ultimately, of actin and myosin (Potter & Gergely, 1974). Clearly, in order to test such models for thin filament regulation, information on the modes of interaction between the regulatory proteins of muscle will be of great importance.

Chemical cross-linking has been used extensively to study the interactions between proteins (Peters & Richards, 1977; Das & Fox, 1979). In particular, Hitchcock (1975), using bifunctional chemical cross-linkers, and Sutoh (1980), using a photochemical cross-linker, have studied the spatial organization among the thin filament proteins. The reagents used in these studies are such that cross-linkers are attached to lysine residues at multiple sites in the proteins. Photochemical cross-linkers whose chemically reactive moiety is specific for sulfhydryl groups have been synthesized (Hixson & Hixson, 1975; Henkin, 1977; Huang & Richards, 1977; Chong & Hodges, 1981a; Moreland et al., 1982). Since specific modification of protein sulfhydryls can be achieved more readily, these reagents permit the probing of protein–protein interactions at specific sites.

In this paper, we report the utilization of 4-maleimidobenzophenone (BP-Mal) as a site-specific photochemical cross-linking reagent. In comparison with other such reagents, this reagent enjoys a number of advantages. First, its maleimide moiety reacts with protein sulfhydryls rapidly and with high specificity. Second, upon photoexcitation, its benzophenone moiety converts to a triplet diradical that is inert toward water molecules in the solvent and reverts to the ground state if no photoreaction takes place. In contrast, the aryl azide moiety of most photochemical cross-linkers irreversibly photoconverts to a nitrene, which is reactive toward water as well as protein moieties. Thus, whereas the cross-linking efficiency of aryl azides is necessarily lowered by an undesirable side reaction with water, the efficiency of benzophenone derivatives can in principle and in practice approach 100% (Campbell & Gioannini, 1978; Williams & Coleman, 1982). Successful utilization of BP-Mal itself has been reported by Tao et al. (1985) and Hardwicke and Szent-Gyorgyi (1985).

Rabbit skeletal $\alpha\alpha$Tm was specifically labeled at Cys-190 with BP-Mal and reconstituted with Tn to form a functional regulatory complex. Irradiation of this complex resulted in the formation of products corresponding to BP-Tm cross-linked to TnT, TnI, and TnC, with decreasing yields of cross-linking in that order. In separate experiments, TnC was labeled at Cys-98 with BP-Mal and reconstituted with either TnI or TnT to form the binary complexes, or with both to form the ternary complex. In confirmation of a preliminary report (Tao et al., 1986), photo-cross-linking of BP-TnC to TnI and TnT took place in both binary complexes as well as in the ternary complex. These findings suggest that all three Tn subunits meet at points near Cys-190 of Tm and Cys-98 of TnC. Comparison of our results with those of Chong and Hodges (1981b, 1982) and the relevance of our findings to current structural models of the regulatory complex will be presented.

Materials and Methods

Materials. BP-Mal was purchased from Molecular Probes (Junction City, OR). Materials for polyacrylamide gel electrophoresis were from Bio-Rad (Richmond, CA). Hepes, DTT, ATP, EDTA, and other reagents for routine analyses were from Sigma (St. Louis, MO).

Protein Preparation. Tn subunits and unfractionated Tm were prepared from rabbit skeletal ether powder as described in Greaser and Gergely (1971). αTm was separated from βTm by (carboxymethyl)cellulose (CM-52; Whatman, Clifton, NJ) chromatography in 8 M urea (Ultra Pure, Schwarz/Mann, Cambridge, MA) according to Cummings and Perry (1973), followed by dialysis to form renatured $\alpha\alpha$Tm. Actin was extracted from rabbit skeletal acetone powder according to Spudich and Watt (1971). Myosin was prepared according to Balint et al. (1975). Chymotryptic S1 was prepared from myosin according to Weeds and Pope (1977).

Protein Labeling. BP-Tm was prepared as follows: $\alpha\alpha$Tm was first reduced with 10 mM DTT for 2 h at 37 °C and isolated by isoelectric precipitation. The reduced $\alpha\alpha$Tm (2 mg/mL) in 20 mM Hepes and 0.1 M NaCl, pH 7.5, was then treated with BP-Mal (added from a 10 mM stock solution in dimethylfomamide) at a molar ratio of 2:1 (reagent to protein) and incubated for 2 h at room temperature. The reaction was quenched with excess DTT and dialyzed to remove excess reagents. The extent of labeling was estimated both by Ellman's assay to measure the amount of unreacted sulfhydryls and by spectrophotometry using ϵ_{280}(BP) = 13 000 M^{-1} cm^{-1} (Tao et al., 1985) and E_{280}(Tm) = 0.24 $(mg/mL)^{-1}$ cm^{-1} (Lehrer, 1975). Protein concentrations of BP-Tm samples were determined by the biuret assay. Typically, 1.2 mol of label was incorporated per mole of Tm dimer (M_r 66 000). Also, the extent of labeling was identical using either method of determination, indicating that the BP labels were incorporated exclusively into the sulfhydryl at Cys-190.

BP-TnC was prepared by using the procedure described in Tao et al. (1985). Typically, 1 mol of BP label was incorporated per mole of TnC.

Tn subunits were radiolabeled with [^{3}H]formaldehyde (100 mCi/mmol; New England Nuclear, Boston, MA) by the method of reductive methylation according to Jentoff and Dearborn (1979). Typically, 0.2–0.4 lysine residue was methylated per mole of protein.

Protein Reconstitution. The binary and ternary Tn complexes were obtained as follows: The individual subunits (labeled or unlabeled) were unfolded in a medium containing 4 M GdmCl, 2 mM EDTA, 5 mM DTT, and 20 mM Hepes, pH 7.5. They were then combined at equimolar proportions and dialyzed against a buffer containing 0.1 M NaCl, 2 mM DTT, 1 mM $CaCl_2$, and 20 mM Hepes, pH 7.5.

Miscellaneous. Photo-cross-linking was carried out in a Rayonet RPR-100 photochemical reactor (Southern New England Ultraviolet, Hamden, CT). Unless otherwise specificed, proteins were irradiated in a medium containing 20 mM Hepes and 0.1 M NaCl, pH 7.5 at 4 °C, 1 mM $CaCl_2$ or 2 mM EGTA for 30 min. Preparation and scintillation counting of gel slices were carried out as described in Tao and Lamkin (1984). Autofluorography was carried out by spraying the dried gel with an autoradiography enhancer (EN^{3}HANCER, New England Nuclear) and exposing it to Kodak X-OMAT AR film. ATPase activity was measured by the pH-stat method using the Radiometer TTT80 system according to White (1982).

Results

Cross-Linking of BP-Tm to Tn Subunits. Rabbit skeletal $\alpha\alpha$Tm labeled at its sulfhydryls at Cys-190 with BP-Mal was reconstituted with the ternary Tn complex and irradiated for 30 min in the photochemical reactor. A variety of cross-linked products were formed, as evidenced by the appearance of bands with lower electrophoretic mobility than that of TnT (Figure 1, lane d or e). To ascertain whether these bands arise from cross-linking between Tm and Tn subunits or from cross-linking between Tm chains, the experiment was carried out with tritium-labeled ternary Tn complex. Autofluorography

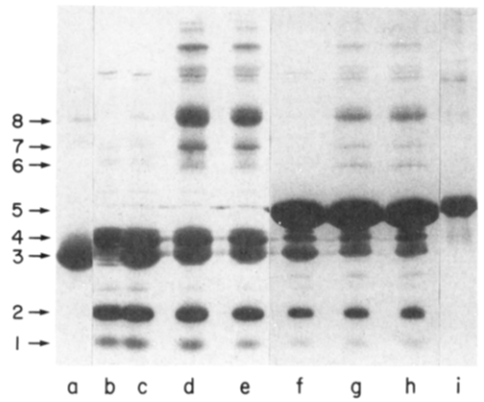

FIGURE 1: SDS–polyacrylamide gel electrophoretogram for BP-Tm cross-linked to the subunits of Tn (7.5–15% gradient running gel, 4% stacking gel). Bands 1–5 are respectively TnC (M_r 17 000), TnI (M_r 21 000), BP-Tm monomer (M_r 33 000), TnT (M_r 30 500), and actin (M_r 42 000); bands 6–8 are identified as the crosslinked products between BP-Tm monomer and TnC, TnI, and TnT, respectively. Lane a, BP-Tm; lane b, the ternary Tn complex; lanes c–e, the Tn·BP-Tm complex before irradiation, irradiated in the presence of 1 mM $CaCl_2$, and irradiated in the presence of 2 mM EDTA, respectively; lanes f–h, the Tn·BP-Tm·F-actin complex before irradiation, irradiated in 1 mM $CaCl_2$, and irradiated in 1 mM EDTA, respectively; lane i, actin. All samples were irradiated for 30 min. All gel samples were run in 2 mM EDTA and 1 mM $CaCl_2$, as the mobility of TnC was found to be Ca^{2+} dependent.

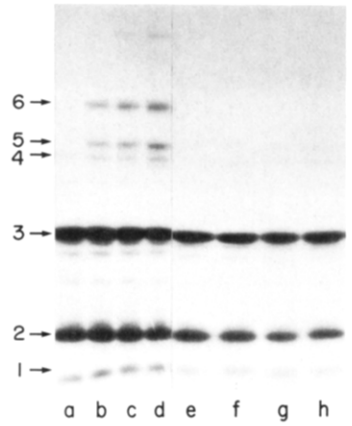

FIGURE 2: Autofluorogram of BP-Tm cross-linked to tritiated Tn, showing the absence of cross-linking in a high-salt dissociating medium. Gel conditions are the same as for Figure 1. Bands 1–3, TnC, TnI, and TnT, respectively. Bands 4–6, cross-linked products between BP-Tm monomer and TnC, TnI and TnT, respectively. Lanes a–d, BP-Tm·[^{3}H]Tn irradiated for 0, 5, 10, and 20 min, respectively, in a medium containing 0.1 M NaCl. Lanes e–h, BP-Tm·[^{3}H]Tn irradiated for 0, 5, 10, and 20 min, respectively, in a medium containing 1 M NaCl.

revealed that with increasing irradiation time, sets of bands appear that correspond to cross-linking between BP-Tm and radiolabeled Tn subunits (Figure 2, lanes a–d). As a control experiment, irradiation was carried out on samples prepared in a medium of higher ionic strength (1 M NaCl), which is known to dissociate the Tn·Tm complex. It can be seen that no cross-linked products were formed under identical conditions of irradiation (Figure 2, lanes e–h). The same cross-linked products were formed when the Tn·BP-Tm complex was irradiated in the presence of actin (Figure 1, lanes f–h). No Ca^{2+}-dependent change in the relative amounts of cross-linked products was apparent either in the absence or in the presence of actin (Figure 1, lane d vs. lane e and lane g vs. lane h).

On the basis of the order of electrophoretic mobilities, we could tentatively assign bands 6, 7, and 8 in Figure 1 (corresponding to bands 4, 5, and 6 in Figure 2) to products

Table I: Yields of Cross-Linking in the Presence and Absence of Ca^{2+}

	cross-linking yield (%)[a]	
	with Ca^{2+}	without Ca^{2+}
BP-Tm·TnT cross-linking band	30 (28)[b]	29 (32)[b]
other TnT cross-linking bands	14	16
un-cross-linked TnT band	56	55
BP-Tm·TnI cross-linking band	12 (19)[b]	19 (20)[b]
other TnI cross-linking bands	3	6
un-cross-linked TnI band	85	75
BP-Tm·TnC cross-linking band	4	2
un-cross-linked TnC band	96	98
BP-TnC·TnI cross-linking band	43	43
un-cross-linked TnI band	37	37
BP-TnC·TnT cross-linking band	21	29
un-cross-linked TnT band	79	71

[a] Obtained from the radioactivity associated with each band divided by the sum of radioactivities associated with each band. [b] Separate determinations using Tn in which all three subunits were tritium labeled.

resulting from crosslinking between one Tm chain (M_r 33 000) to, respectively, 1 mol of TnC (M_r 18 000), TnI (M_r 21 000), and TnT (M_r 30 500). It should be noted that in separate experiments we have found that Tm dimer (M_r 66 000) produced either by irradiation of BP-Tm or by disulfide cross-linking of the two chains has an electrophorectic mobility similar to that of band 8 in Figure 1 (M. Lamkin and T. Tao, unpublished results). It is, however, dangerous to identify bands on the basis of electrophoretic mobility alone because Tm, and perhaps TnT as well, appears to have anomalous electrophoretic mobilities in SDS (note that although Tm monomer is higher in molecular weight than TnT, its mobility is higher). In order to identify the bands with more confidence, we carried out the experiments using Tn complexes in which each of the subunits was tritium labeled. For example, in experiments using Tn containing [^{3}H]TnT, a major radiolabeled-containing band with the same relative mobility as that of band 8 in Figure 1 was produced upon irradiation (Figure 3, panels b), showing unequivocably that this band corresponds to a cross-linked product between Tm and [^{3}H]TnT. The mobility of the band is such that this cross-linked product most likely contains 1 mol of Tm monomer and 1 mol of TnT. Similarly, using Tn that contained [^{3}H]TnI, we verified the formation of a cross-linked product composed of 1 mol of Tm monomer and 1 mol of TnI (Figure 3, panels c). Finally, using Tn that contained [^{3}H]TnC, we found the formation of a small amount of the cross-linked product containing 1 mol of Tm monomer and 1 mol of TnC (Figure 3, panels d).

We noted that cross-linked products of higher molecular weights were formed. Many possibilities exist for the composition of these products, including two Tm monomers and one TnT, or one Tm monomer, one TnT, and one TnI, or one Tm monomer, one TnT, and one TnC. No attempts were made to identify these products.

The yields of cross-linking were obtained by summing the amount of radioactivity associated with each gel band. As shown in Table I, the yield is highest for cross-linking between BP-Tm and TnT and intermediate for BP-Tm and TnI followed by a small but reproducible amount of cross-linking between BP-Tm and TnC. Within experimental error, we found no change in the yield of cross-linking between BP-Tm and TnT as a function of Ca^{2+}. For TnI and TnC, there might be small Ca^{2+}-dependent changes in the cross-linking yields, but the uncertainties in the measurements are too large (owing to the low yields) to permit a definitive statement.

The functional activity of BP-Tm was investigated. Table II shows that the capacity of BP-Tm for inhibiting acto–S1

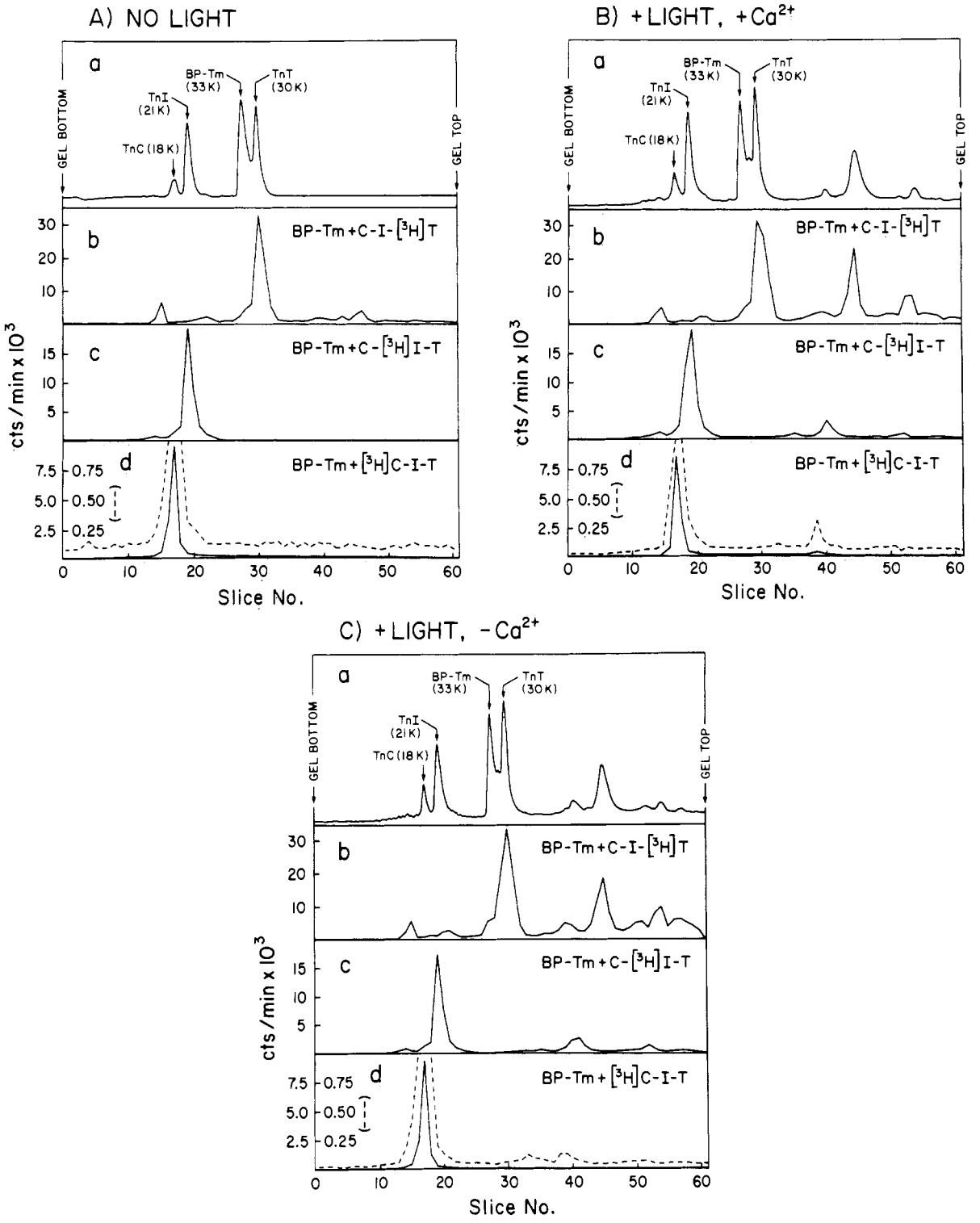

FIGURE 3: Densitometric and radioactivity scans of gels for BP-Tm cross-linking to Tn subunits, under the following conditions: (A) before irradiation; (B) irradiated in the presence of 1 mM $CaCl_2$; (C) irradiated in the presence of 2 mM EDTA. Gel conditions are the same as those for Figure 1. Panels a, densitometric scans of Coomassie Blue stained gels. Panels b–d, radioactivity scans of gels for BP-Tm complexed to reconstituted ternary Tn complex containing tritiated TnT, TnI, TnC, respectively. The dotted curves in panels d are the same as the solid curves but plotted at a scale that is expanded 10-fold.

ATPase is somewhat reduced when compared to unmodified Tm. Sedimentation experiments showed that this is due to a reduction in the affinity of BP-Tm for actin. In the presence of Tn, however, the reconstituted regulatory system retains its capacity to stimulate and inhibit acto-S1 ATPase in the presence and absence of Ca^{2+}, respectively. The same results were obtained when either TnI or TnC was tritiated. These findings show that the complexes used in the cross-linking experiments function essentially identically with the complex formed with unmodified Tm and Tn.

Cross-Linking of BP-TnC to TnI and TnT. Previously, we have reported that BP-TnC can be photo-cross-linked to TnI in the BP-TnC·TnI binary complex and to TnT in the BP-TnC·TnT binary complex (Tao et al., 1986). For the BP-TnC ternary complex, at least two products were formed, one with the same mobility as the cross-linking product produced by irradiating BP-TnC·TnI. Other bands appear with mobilities that are in the same range as that of the product formed by

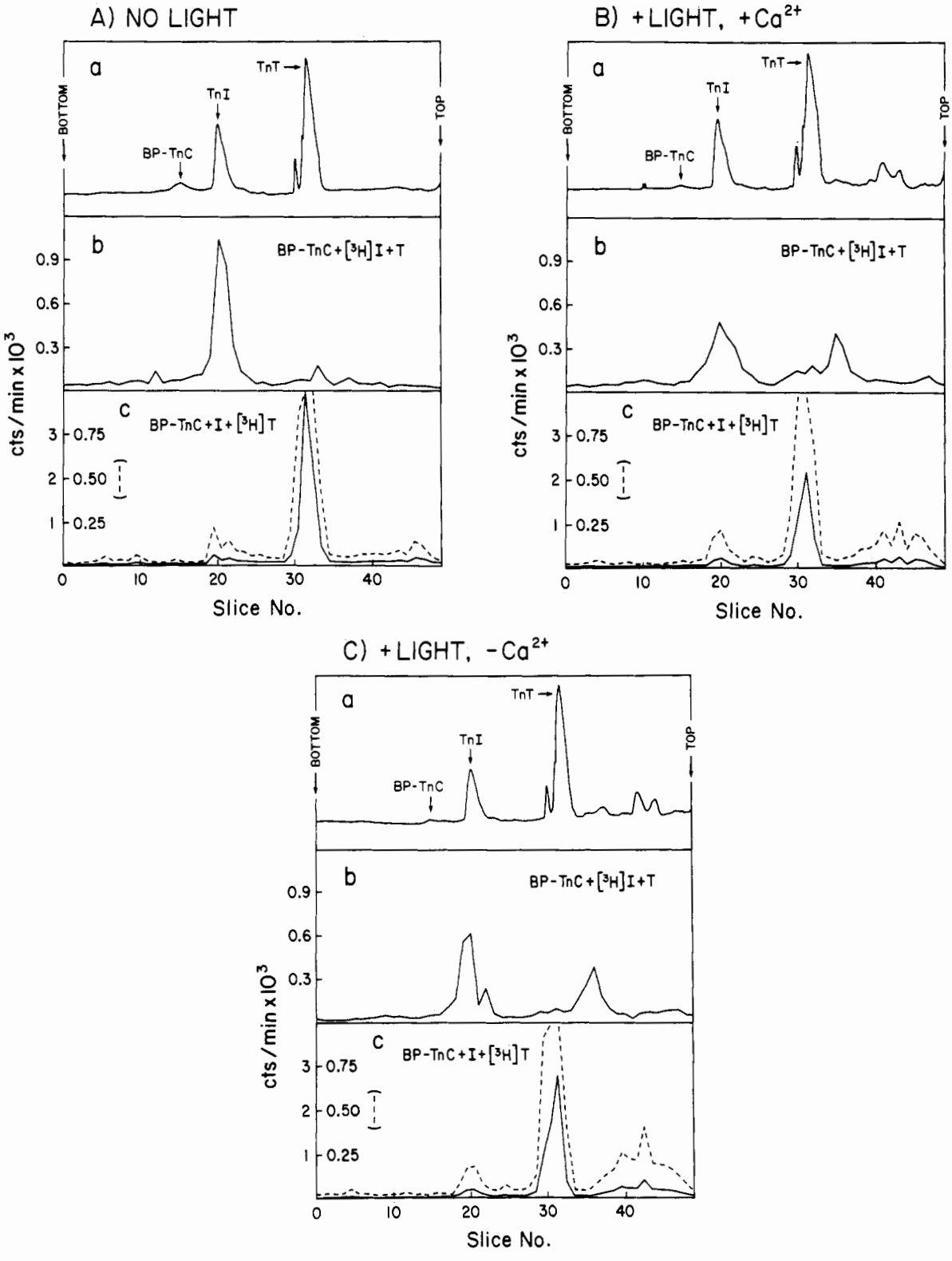

FIGURE 4: Densitometric and radioactivity scans of gels for BP-TnC cross-linking to tritiated TnI and TnT under the following conditions: (A) before irradiation; (B) irradiated in the presence of 1 mM $CaCl_2$; (C) irradiated in the presence of 2 mM EDTA. Panels a, densitometric scans of Coomassie Blue stained gels. Panels b and c, radioactivity scans of gels for BP-TnC·[^{3}H]TnI·TnT and for BP-TnC·TnI·[^{3}H]TnT, respectively. The dotted curves in panels c are the same as the solid curves but plotted at a scale that is expanded 4-fold.

irradiating the BP-TnC·TnT complex (Tao et al., 1986). In control experiments, the complexes were irradiated in a medium containing 6 M urea and 2 mM EDTA, a condition that is known to dissociate the complexes. No cross-linked products were formed, indicating that nonspecific cross-linking via random collisions between the proteins was absent. Also, there was no difference in the cross-linking pattern when irradiation times were shortened to a minimum of 5 min.

To positively identify the cross-linked products, [^{3}H]TnI was reconstituted with BP-TnC and unlabeled TnT to form the BP-TnC·[^{3}H]TnI·TnT complex. Irradiation of this material produced a single radiolabeled band resulting from cross-linking between BP-TnC and [^{3}H]TnI (Figure 4, panels b). Similarly, using [^{3}H]TnT, irradiation of the BP-TnI·[^{3}H]TnT complex produced at least two bands, corresponding to products arising from cross-linking between BP-TnC and

Table II: ATPase Activities of Myosin Subfragment 1 in the Presence of Reconstituted Thin Filaments: Effects of Benzophenone Labeling on Tm and TnC and Reductive Methylation of Tn Subunits[a]

sample	ATPase act. [mol of ATP (mol of S1)$^{-1}$ s^{-1}]
S1	0.04
S1 + F-actin	0.36
S1 + F-actin + Tm	0.20
S1 + F-actin + Tm + Tn + Ca^{2+}	0.36
S1 + F-actin + Tm + Tn + EGTA	0.12
S1	0.06
S1 + F-actin	0.43
S1 + F-actin + BP-Tm	0.30
S1 + F-actin + BP-Tm + C–I*–T + Ca^{2+}	0.36
S1 + F-actin + BP-Tm + C–I*–T + EGTA	0.22
S1	0.05
S1 + F-actin	0.47
S1 + F-actin + BP-Tm	0.35
S1 + F-actin + BP-Tm + C*–I–T + Ca^{2+}	0.38
S1 + F-actin + BP-Tm + C*–I–T + EGTA	0.16
S1[b]	0.06
S1 + F-actin	0.43
S1 + F-actin + Tm	0.23
S1 + F-actin + Tm + Tn + Ca^{2+}	0.39
S1 + F-actin + Tm + Tn + EGTA	0.15
S1	0.05
S1 + F-actin	0.32
S1 + F-actin + Tm	0.20
S1 + F-actin + Tm + BP-TnC·TnI·TnT + Ca^{2+}	0.33
S1 + F-actin + Tm + BP-TnC·TnI·TnT + EGTA	0.10

[a] Rates of ATP hydrolysis were determined from pH-stat traces. [S1] = 3.6 μM; [F-actin] = 3.8 μM; [Tm] = 0.49 μM; [Tn] = 0.6 μM; [Ca^{2+}] = 0.1 mM; [EGTA] = 2 mM; in 30 mM NaCl and 5 mM $MgCl_2$, pH 7.9, 23 °C. C* and I* are formyl-methylated TnC and TnI, respectively. [b] A new batch of S1 was used for this and the ensuing measurements.

[^{3}H]TnT (Figure 4, panels c). No large difference in either the pattern (Figure 4) or the yields (Table I) of cross-linking was detectable as a function of Ca^{2+}.

In the fully reconstituted system containing S1, actin, Tm, and Tn, we found that the BP-TnC·TnI·TnT complex functions well as TnC·TnI·TnT in regulating the Mg^{2+}-ATPase activity of the system as a function of Ca^{2+} (Table II), showing that the activity of TnC is not impaired by modification at Cys-98 with BP-Mal.

Discussion

Our results show that all three Tn subunits can be cross-linked to BP-Tm. This cross-linking cannot be due to nonspecific interactions via random collisions between Tn·BP-Tm complexes because under conditions that dissociate the Tn·BP-Tm complex, no cross-linking occurred (Figure 2). The fact that the same sets of cross-linking bands were obtained at irradiation times as short as 5 min also argues against the occurrence of cross-linking due to collisonal interactions (Figure 2). Also, the observed cross-linking cannot be due to the formation of dimeric Tn·BP-Tm because the same cross-linking pattern was obtained in the presence of actin, when the formation of Tn·BP-Tm dimers is not possible (Figure 1).

Our finding that BP-Tm cross-links to all three subunits of Tn shows that some portion of each subunit is within 10 Å (the approximate distance between the attachment points of the cross-linker) of Cys-190 of Tm. The yields of cross-linking are ~45% for TnT, 12–20% for TnI, and ~3% for TnC. In general, cross-linking yields depend on whether there are protein moieties nearby, and whether these protein moieties possess the appropriate chemical properties. In this case here, the BP label is known to nonselectively photoreact with carbon–hydrogen bonds of any protein moiety (Walling & Gibian, 1965), so that cross-linking yields are determined primarily by the distance between the protein and the photolabel. The large cross-linking yield for TnT can therefore be interpreted to indicate that some portion of the TnT molecule is in close proximity to and perhaps in contact with Cys-190 of Tm. The lesser cross-linking yields for TnI and TnC indicate that although these two proteins are in the proximity of Cys-190 of Tm, they are probably not in contact with it.

Our finding that TnT can be cross-linked to BP-Tm is in agreement with previous cross-linking studies of Chong and Hodges (1982), who used the cleavable photo-cross-linker AGTC attached at Cys-190 of Tm. However, Chong and Hodges did not find cross-linking to TnI nor TnC. This discrepancy could be due to the difference in the nature of the photolabels in the photo-cross-linkers used. It is known that photolysis of the BP moiety in BP-Mal produces the triplet diradical intermediate which does not react with water and which reverts to the ground state if no reactions with protein moieties take place (Helene, 1972; Ledger & Porter, 1972). In contrast, photolysis of the phenyl azide moiety in AGTC is known to irreversibly produce the nitrene radical which will react with water if no protein moieties are nearby (Bayley & Knowles, 1977). Our results can therefore be reconciled with those of Chong and Hodges as follows: TnT makes substantial contact with Cys-190 of Tm, so that both the BP and the phenyl azide photolabels are shielded by protein moieties in TnT from collisions with water molecules, resulting in substantial photo-cross-linking in both cases. TnI and TnC, on the other hand, are not in contact with Cys-190 of Tm, so that the photolabels collide with water molecules far more frequently than with protein moieties in TnI and TnC. For the BP label, this would still produce the small amount of cross-linking that we observed. For the phenyl azide label, however, competition with water can be so severe that cross-linking with protein moieties may become undetectable.

Our findings differ from those of Chong and Hodges (1982) in another respect; while they found that the Tm-TnT cross-linking yield was Ca^{2+}-dependent, we did not. It is possible that this is due to the relatively more selective nature of the phenyl azide moiety compared to the BP moiety, so that a small change in the environment around the phenyl azide label can cause a larger change in cross-linking efficiency.

We found that BP-TnC cross-links with both TnI and TnT in the binary as well as in the ternary complexes. Again, this cross-linking cannot be due to random collisions between the proteins since no photo-cross-linking occurred under conditions that dissociate the complexes. That BP-TnC cross-links to TnI is in agreement with Chong and Hodges (1981b), who found that TnC labeled with AGTC at Cys-98 cross-links with TnI. It is also in support of various other workers' suggestions that the Cys-98 region of TnC forms part of the TnC–TnI interaction site (Dalgarno et al., 1982; Cachia et al., 1983; Leavis et al., 1984).

Our results show that TnT interacts with TnC at or near Cys-98 of the latter both in the binary and in the ternary complex. Further, we noted that whereas irradiation of the BP-TnC·TnT binary complex produced a single cross-linking band, irradiation of the ternary complex produced several cross-linking bands that are somewhat different in electrophoretic mobility from each other, and from the binary complex cross-linking band. Previously, we have found that the

lifetime and quenching rate constants of a fluorescence probe attached at Cys-98 of TnC are different in the binary complexes compared to those in the ternary complex (Leavis et al., 1984). Taken together, both findings suggest that the interaction between TnT and TnC in the ternary complex is different from that in the binary complex. The prescence of multiple photo-cross-linking bands suggests that in the ternary complex the Cys-98 region of TnC is not in direct contact with TnT, such that the attached BP label can photo-cross-link at several sites with TnT. Consistent with our fluorescence studies (Leavis et al., 1984), we found no apparent change in either the cross-linking pattern or the cross-linking yields as a function of Ca^{2+}, indicating that there are no large-scale Ca^{2+}-dependent structural rearrangements in the Cys-98 region of TnC for the ternary Tn complex.

Generally speaking, our findings are consistent with the picture that the three subunits of Tn form extensive contacts with each other (Leavis & Gergely, 1984). In particular, our results show that all three subunits meet at points near Cys-98 of TnC and Cys-190 of Tm. On the basis of electron microscopy and other studies, a model has been proposed in which an elongated TnT molecule interacts lengthwise with Tm, while TnC and TnI are depicted as globular moieties that interact with each other and with the carboxyl-terminal region of TnT (Ohtsuki, 1979; Mak & Smillie, 1981; Flicker et al., 1982). Our results are consistent with this model and predict that Cys-90 of Tm and Cys-98 of TnC are located at interstitial regions between TnC, TnI, and TnT.

In conclusion, we found that BP-Mal serves admirably as a site-specific photochemical cross-linker for studying the interactions between muscle proteins. The high reactivity of the maleimide moiety is such that nearly 100% sulfhydryl labeling was achieved. The high photo-cross-linking efficiency of the BP label allows us to detect and characterize interactions that may not be evident using other cross-linkers. Since radiolabeled BP-Mal has been synthesized (Tao et al., 1985), it will be possible to carry out peptide and amino acid analyses of the cross-linked products. Our future work using BM-Mal will be directed towards biochemical characterization of the cross-linked materials and localization of the cross-linking sites.

ACKNOWLEDGMENTS

We thank Drs. John Gergely and Sherwin S. Lehrer for their support and interest in this work.

Registry No. BP-Mal, 92944-71-3; ATPase, 9000-83-3; L-Cys, 52-90-4.

REFERENCES

Balint, M., Sreter, F. A., Wolf, I., Nagy, B., & Gergely, J. (1975) *J. Biol. Chem. 250*, 6168–6177.

Bayley, H., & Knowles, J. 1977) *Methods Enzymol. 46*, 69–114.

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

Chong, P. C. S., & Hodges, R. S. (1981a) *J. Biol. Chem. 256*, 5064–5070.

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

Chong, P. C. S., & Hodges, R. S. (1982) *J. Biol. Chem. 257*, 9152–9160.

Cummings, P., & Perry, S. V. (1973) *Biochem. J. 133*, 765–777.

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.

Das, M., & C. F. (1979) *Annu. Rev. Biophys. Bioeng. 8*, 165–193.

Ebashi, S., Endo, M., & Ohtsuki, I. (1969) *Q. Rev. Biophys. 2*, 351–384.

Flicker, P. F., Phillips, G. N., Jr., & Cohen, C. (1982) *J. Mol. Biol. 162*, 495–501.

Greaser, M., & Gergely, J. (1971) *J. Biol. Chem. 246*, 4226–4233.

Hardwicke, P. M. D., & Szent-Gyorgyi, A. G. (1985) *J. Mol. Biol. 183*, 203–211.

Helene, C. (1972) *Photochem. Photobiol. 16*, 519–522.

Henkin, J. (1977) *J. Biol. Chem. 252*, 4293–4297.

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

Hixson, S. H., & Hixson, S. S. (1975) *Biochemistry 14*, 4251–4254.

Huang, C.-K., & Richards, F. M. (1977) *J. Biol. Chem. 252*, 5514–5521.

Huxley, H. E. (1972) *Cold Spring Harbor Symp. Quant. Biol. 37*, 361–376.

Jentoft, N., & Dearborn, D. G. (1979) *J. Biol. Chem. 254*, 4359–4365.

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

Leavis, P. C., Gowell, E., & Tao, T. (1984) *Biochemistry 23*, 4156–4161.

Ledger, M. B., & Porter, G (1972) *J. Chem. Soc., Faraday Trans. 1 68*, 539–553.

Lehrer, S. S. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3377–3381.

Mak, A. S., & Smillie, L. B. (1981) *J. Mol. Biol. 149*, 541–550.

Moreland, R. B., Smith, P. K., Fujimoto, E. K., & Dockter, M. E. (1982) *Anal. Biochem. 121*, 321–326.

Ohtsuki, I. (1979) *J. Biochem. (Tokyo) 86*, 491–497.

Peters, K., & Richards, F. M. (1977) *Annu. Rev. Biochem. 46*, 523–551.

Potter, J. D., & Gergely, J. (1974) *Biochemistry 13*, 2697–2703.

Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866–4871.

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

Tao, T., & Lamkin, M. (1984) *FEBS Lett. 168, 169*–173.

Tao, T., Lamkin, M., & Scheiner, C. J. (1984) *Biophys. J. 45*, 261a.

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

Tao, T., Gergely, J., & Leavis, P. C. (1986) *Biophys. J. 49*, 142–143.

Walling, C., & Gibian, M. (1965) *J. Am. Chem. Soc. 87*, 3361–3364.

Weeds, A. G., & Pope, B. (1977) *J. Mol. Biol. 111*, 129–157.

White, H. D. (1982) *Methods Enzymol. 85*, 698–708.