served), multiplied by y, the fraction of the ϵ-NH_2 that is unprotonated. It is easy to show that $y = 1/[1 + \exp[a(pK - pH)]]$, where $a = 2.303....$ When pH is much less than pK [i.e., $a(pK - pH)$ is large], the exponential is much larger than unity, and $k \simeq k_i \exp[a(pH - pK)]$, whereupon

$$\log k = pH - (pK - \log k_i)$$

Since in the enzyme cases k_i is not precisely known (the plateau value), pK cannot be obtained from the intercept of this plot, but when log k is plotted against pH, a straight line of slope unity should be obtained. This was exactly so for N^{α}-Cbz-lysine and poly-L-lysine; for k_f and k_s, however, slopes of 0.7 and 0.61, respectively, were obtained (Figure 7). There is thus a further anomaly in the ionization curves of the enzyme ϵ-NH_2. In the case of the model compounds, the plateau levels could be measured, and the intercepts gave pK - log k_i values of 7.4 and 6.4, respectively—in good aggrement with estimates from Figures 5 and 6.

References

Applequist, J., & Doty, P. (1962) in *Polyamino Acids, Polypeptides and Proteins* (Stahmann, M. A., Ed.) p 161, University of Wisconsin Press, Madison, WI.

Cascone, O., Bonino, B. J. M., Paladini, A. C., & Santome, J. A. (1977) *Arch Biochem. Biophys. 180*, 303-309.

Fabian, F., & Muhlrad, A. (1968) *Biochim. Biophys. Acta 162*, 596-603.

Fiske, C. H., & Subbarow, Y. (1929) *J. Biol. Chem. 66*, 375-400.

Freedman, R. B., & Radda, G. K. (1968) *Biochem. J. 108*, 383-391.

Goldfarb, A. R. (1966a) *Biochemistry 5*, 2570-2574.

Goldfarb, A. R. (1966b) *Biochemistry 5*, 2574-2578.

Haynes, R., Osuga, D. T., & Feeney, R. E. (1967) *Biochemistry 6*, 541-547.

Hirs, C. H. W. (1962) *Brookhaven Symp. Biol. 15*, 154-183.

Hozumi, T., & Muhlrad, A. (1981) *Biochemistry 20*, 2945-2950.

Kitagawa, S., Yoshimura, J., & Tonomura, Y. (1961) *J. Biol. Chem. 236*, 902-906.

Kubo, S. Tokura, S., & Tonomura, Y. (1960) *J. Biol. Chem. 233*, 2835-2839.

Miyanishi, T. (1980) Japan-U.S. Seminar, Sapporo, Japan.

Muhlrad, A. (1968) *Biochim. Biophys. Acta 162*, 444-451.

Muhlrad, A., & Fabian, F. (1970) *Biochim. Biophys. Acta 216*, 422-427.

Muhlrad, A., & Afolayan, A. (1975) *J. Mechanochem. Cell Motil. 3*, 99-101.

Muhlrad, A., Lamed, R. R., & Oplatka, A. (1975) *J. Biol. Chem. 250*, 175-181.

Muhlrad, A., Oplatka, A., & Lamed, R. (1976) *Biochim. Biophys. Acta 452*, 227-238.

Okuyama, T., & Satake, K. (1960) *J. Biochem. (Tokyo) 47*, 454-466.

Shimizu, T. (1979) *J. Biochem. (Tokyo) 85*, 1421-1426.

Srivastava, S. K., Tonomura, Y., & Inoue, A. (1979) *J. Biochem. (Tokyo) 86*, 725-31.

Takashi, R., Muhlrad, A., Hozumi, T., & Botts, J. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 1936.

Tonomura, Y., Yoshimura, J., & Onishi, T. (1963) *Biochim. Biophys. Acta 78*, 698-704.

Tonomura, Y., Appel, P., & Morales, M. F. (1966) *Biochemistry 5*, 515-521.

Tseng, Y. W., & Yang, J. T. (1977) *Biopolymers 16*, 921-935.

Weeds, A. G., & Taylor, R. S. (1975) *Nature (London) 257*, 54-56.

Synthesis and Application of Cleavable Photoactivable Heterobifunctional Reagents†

Elio F. Vanin and Tae H. Ji*

ABSTRACT: Three different cleavable photoactivable heterobifunctional reagents have been synthesized and characterized by proton NMR, ^{13}C NMR, mass spectroscopy, infrared spectroscopy, and thin-layer chromatography. They are the *N*-hydroxysuccinimide ester of 3-[(4-azidophenyl)dithio]-propionic acid, methyl 3-[(4-azidophenyl)dithio]propionimidate, and *N*-[(4-azidophenyl)thio]phthalimide. Concanavalin A was coupled with one of the reagents, the *N*-hydroxysuccinimide ester of 3-[(4-azidophenyl)dithio]propionic acid, and radioiodinated. The [^{125}I]lectin derivative and [^{125}I]lectin showed similar specific binding to the receptors on the human erythrocyte membrane. Upon photolysis, subunits of the [^{125}I]lectin derivative were cross-linked to produce dimer, trimer, and tetramer. These cross-linked complexes were readily cleaved by reducing the disulfide bond of the reagent.

Chemical cross-linking has been a major tool for predicting subunit structures of molecules and molecular associations in cell membranes [for reviews, see Peters & Richards (1977) and Ji (1979)]. Recently its application has been extended to the identification of surface receptors for macromolecular ligands (Ji, 1976). In this case, photoactivable heterobifunctional reagents are coupled to macromolecular ligands via the nonphotosensitive chemically reactive group, and the ligand derivative is cross-linked to receptors by photoactivation after binding. Therefore, these reagents can be used effectively to probe membrane receptors and their environment in intact cells.

To date most of the available photoactivable heterobifunctional reagents used for receptor studies are noncleavable.

† From the Department of Biochemistry, University of Wyoming, Laramie, Wyoming 82071. *Received May 18, 1981.* This work was supported by Grant GM-23586 from the National Institutes of Health.

0006-2960/81/0420-6754$01.25/0

As a result, cross-linked ligand–receptor complex cannot be separated, and the identity of the receptor must be implied indirectly. This indirect prediction is certainly suspect because of a number of drawbacks (Ji, 1979). The use of cleavable reagents (Jaffe et al., 1980) together with two-dimensional gel electrophoresis can greatly facilitate the identification of membrane receptors. The lack of availability of such reagents has therefore limited the use of macromolecular affinity labeling as a method for the identification of membrane receptors. Earlier, we have discussed a few of such reagents (Kiehm & Ji, 1977; Ji, 1979). Here we describe a comprehensive synthesis and characterization of these reagents as well as new ones.

Experimental Procedures

Synthesis of 4,4′-Azidodiphenyl Disulfide. All reagents were purchased from Aldrich Chemical Co. and recrystallized or distilled before use. Due to light sensitivity, all reactions involving the 4-azidophenyl group were carried out in subdued red light. 4,4′-Azidodiphenyl disulfide was prepared in two steps. First, 4,4′-aminodiphenyl disulfide was converted to the corresponding diazonium salt by dropwise addition of $NaNO_2$ (110 mmol in 40 mL of H_2O) over a period of 20 min, with vigorous stirring, to a suspension of 4,4′-aminodiphenyl disulfide (50 mmol in 500 mL of 2 M HCl) at 0 °C. The reaction was allowed to proceed for 10 min. The presence of the diazonium salt was confirmed by using β-naphthol in 0.5 M NaOH. The 4,4′-azidodiphenyl disulfide was formed by dropwise addition of NaN_3 (120 mmol in 60 mL of H_2O) to the diazonium salt solution over a period of 20 min at 0 °C with vigorous stirring. The reaction mixture was then incubated for a further 10 min at 0 °C and then placed at −15 °C overnight. The resulting precipitate was collected, washed with 2 L of H_2O, and dried.

The 4,4′-azidodiphenyl disulfide was recrystallized by using petroleum ether (bp 35–70 °C) to give an overall yield of 31 mmol (62%). The crystallized compound had the following properties: R_f [benzene/pentane (1:4 v/v)] 0.54; λ_{max} 266 nm (ε 45 800); IR 2140 (N_3) and 1600 cm^{-1} (aromatic); proton NMR δ 6.8 (aromatic, 2 H, d) and 7.35 (aromatic, 2 H, d); mass spectrum, *m/z* 300 (parent molecular ion).

Synthesis of N-[(4-Azidophenyl)thio]phthalimide. The *N*-[(4-azidophenyl)thio]phthalimide was synthesized according to the procedure of Behforouz & Kerwood (1968). Chlorine gas was bubbled into a solution of 4,4′-azidodiphenyl disulfide (25 mmol in 30 mL of dry pentane) at 0 °C and stirred until the weight increased by 2.45 g (37.5 mmol of chlorine). After standing for 60 min at 0 °C, this reaction mixture was added, with stirring, to a solution containing 50 mmol of phthalimide and 60 mmol of triethylamine in 200 mL of dry dimethylformamide. After incubation for 60 min at 0 °C, 2 L of ice water was added and the precipitate collected by filtration and dried. The compound was recrystallized with ethyl acetate to give a final yield of 7.7 g (52%): R_f (benzene) 0.35; mp 144–145 °C dec; λ_{max} 267 nm (ε 19 800); IR 2140 (N), 1700 (carbonyl), 1590 cm^{-1} (aromatic); mass spectrum, *m/z* 296 (parent molecular ion).

Synthesis of 3-[(4-Azidophenyl)dithio]propionic Acid. The 3-[(4-azidophenyl)dithio]propionic acid was synthesized by using a modification of the procedure of Harpp et al. (1970). *N*-[(4-Azidophenyl)thio]phthalimide (40 mmol) was dissolved in 70 mL of benzene, and the 3-mercaptopropionic acid (50 mmol) was added. The reaction mixture was then refluxed, with stirring, for 14 h. The solution was placed at −15 °C overnight in order to precipitate any residual phthalimide still in solution; the majority of the phthalimide, which is insoluble in benzene, precipitated during the refluxing. The reaction mixture was then thawed and filtered, and the resulting filtrate was made 1% with respect to both ethyl acetate and acetic acid. This was then loaded onto a silicic acid column (5 × 45 cm) and the compound eluted with ethyl acetate/acetic acid/benzene (1:1:100 v/v/v), while 10-mL fraction were collected. An aliquot of each fraction was then analyzed by TLC using ethyl acetate/acetic acid/benzene (25:1:75 v/v/v) as the solvent. The fractions containing the acid (R_f 0.32) were pooled and evaporated to dryness, and the residue was recrystallized from petroleum ether (bp 60–110 °C); a yield of 17.6 mmol (43.9%) was obtained. The 3-[(4-azidophenyl)dithio]propionic acid had the following properties: R_f [ethyl acetate/acetic acid/benzene (25:1:75 v/v/v)] 0.32; mp 61.5–63 °C; λ_{max} 264 nm (ε 18 710); IR 2120 (N), 1710 (carbonyl), 1590 cm^{-1} (aromatic); proton NMR δ 6.92 (aromatic, 2 H, d), 7.48 (aromatic, 2 H, d), 2.83 (CH_2CH_2, 4 H, q), and 11.7 (COOH, 1 H, s); ^{13}C NMR δ 32.75 (aliphatic CH_2, 1 C) 33.63 (aliphatic CH_2, 1 C), 119.76 (unsubstituted aromatic, 2 C), 130.16 (unsubstituted aromatic, 2 C), 133.14 (aromatic C–S, 1 C), 139.54 (aromatic C–N_3, 1 C), and 178.12 (COOH, 1 C); mass spectrum, *m/z* 255 (parent molecular ion).

Synthesis of 2-[(4-Azidophenyl)dithio]acetic Acid. As before *N*-[(4-azidophenyl)thio]phthalimide was used as one of the starting materials.

N-[(4-Azidophenyl)thio]phthalimide (30 mmol) was added to a solution of 2-mercaptoacetic acid (36 mmol) in 70 mL of benzene. After being refluxed for 12 h, the solution was kept overnight at −15 °C in order to precipitate any phthalimide still in solution. The thawed solution was made 1% in both ethyl acetate and acetic acid, after which it was loaded onto a silicic acid column (5.5 × 42 cm) and eluted with ethyl acetate/acetic acid/benzene (1:1:100 v/v/v). Ten-milliliter fractions were collected, and each fraction was chromatographed on analytical TLC using ethyl acetate/acetic acid/benzene (20:1:80 v/v/v). The appropriate fractions were then pooled and evaporated to dryness, and the residue was recrystallized from petroleum ether (bp 60–110 °C) to give an overall yield of 8.92 mmol (30%). Compound synthesized in this way had the following properties: R_f [ethyl acetate/acetic acid/benzene (20:1:80 v/v/v)] 0.37; IR 2120 (N_3), 1670 (carbonyl), and 1600 cm^{-1} (aromatic); proton NMR δ 3.44 (CH_2, 2 H, s), 6.94 (aromatic, 2 H, d), 7.52 (aromatic, 2 H, d), and 10.89 (COOH, 1 H, s); ^{13}C NMR δ 40.47 (CH_2, 1 C), 119.73 (unsubstituted aromatic, 2 C), 131.63 (unsubstituted aromatic, 2 C), 132.08 (aromatic, C–S, 1 C), 140.28 (aromatic C–N_3), and 175.34 (COOH, 1 C); mass spectrum, *m/z* 240 (parent molecular ion).

Synthesis of the N-Hydroxysuccinimide Ester of 3-[(4-Azidophenyl)dithio]propionic Acid. 3-[(4-Azidophenyl)dithio]propionic acid (10 mmol) was dissolved in 50 mL of dry acetonitrile, and to this NHS[1] (12.5 mmol) in 12.5 mL of dry acetonitrile was added. The coupling was achieved by the addition of 13 mL of a 1 M solution of DCC (13 mmol) in dry acetonitrile. The reaction mixture was then incubated at room temperature for 16 h, and the precipitated dicyclohexylurea was separated from the reaction mixture by centrifugation. The supernatant was saved, and the pellet was washed with 30 mL of dry acetonitrile and recentrifuged. The

[1] Abbreviations used: Con A, concanavalin A; DCC, dicyclohexylcarbodiimide; NHS, *N*-hydroxysuccinimide; APDP, 3-[(4-azidophenyl)dithio]propionic acid; APDA, 2-[(4-azidophenyl)dithio]acetic acid; NHS-APDP, *N*-hydroxysuccinimide ester of 3-[(4-azidophenyl)dithio]propionic acid; MADP, methyl 3-[(4-azidophenyl)dithio]propionimidate; mm, methyl α-D-mannoside.

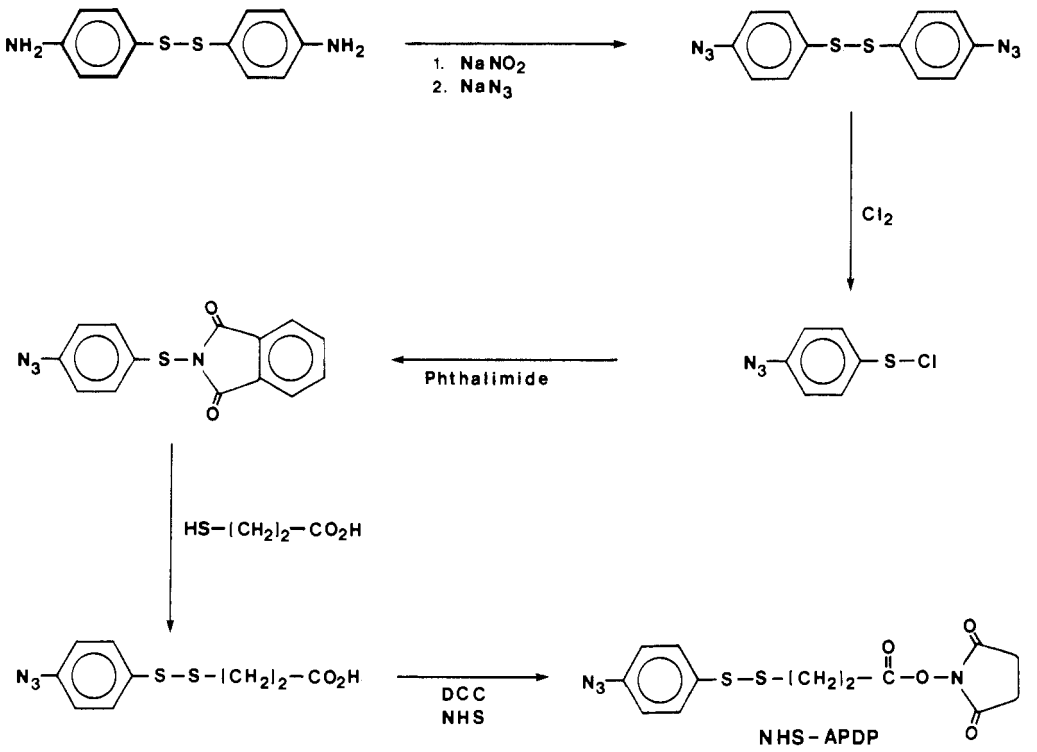

FIGURE 1: Scheme for the synthesis of the cleavable, photosensitive, heterobifunctional reagent NHS-APDP.

pooled supernatants were then evaporated to dryness, and the residue was recrystallized from petroleum ether (bp 30–75 °C) to give a 6.8 mmol (68%) yield. The *N*-hydroxysuccinimide ester of 3-[(4-azidophenyl)dithio]propionic acid had the following properties: R_f (ethyl acetate/acetic acid/benzene (25:1:75 v/v/v)] 0.58; λ_{max} 263 nm (ϵ 19488); IR 2120 (N_3), 1810 and 1780 (NHS carbonyls), and 1740 cm^{-1} (acid carbonyl); proton NMR δ 2.76 (NHS CH_2CH_2, 4 H, s), 2.96 (propionyl CH_2CH_2, 4 H, s), 6.95 (aromatic, 2 H, d), and 7.5 (aromatic, 2 H, d); ^{13}C NMR δ 25.66 (NHS, 2 C), 31.01 (aliphatic CH_2, 1 C), 32.62 (aliphatic CH_2, 1 C), 119.96 (unsubstituted aromatic, 2 C), 130.26 (unsubstituted aromatic, 2 C), 132.92 (aromatic C–S, 1 C), and 139.82 (aromatic C–N_3, 1 C); mass spectrum, *m/z* 352 (parent molecular ion).

Synthesis of 3-Mercaptopropionitrile. The procedure was similar to that of Traut et al. (1973). 3-Chloropropionitrile (370 mmol) was added to thiourea (500 mmol) in 250 mL of H_2O, and the mixture was refluxed for 2 h at 120 °C. After the reaction mixture was cooled to room temperature, it was added to 500 mL of cold acetone, and the resulting precipitate, 3-isothioureidopropionitrile, was collected by filtration to give an overall yield of 330 mmol (90%).

All of the following steps were performed under an atmosphere of nitrogen in order to ensure minimal sulfhydryl oxidation upon the conversion of the 3-isothioureidopropionitrile to 3-mercaptopropionitrile. 3-Isothioureidopropionitrile (0.28 mmol) was dissolved in 60 mL of degassed H_2O, mixed with 28 mL of 50% NaOH (w/v), and then incubated for 30 min at 50 °C. After the mixture was cooled to room temperature, 80 mL of diethyl ether was added and stirred for 5 min. The ether layer was then removed and the aqueous layer extracted once more with 80 mL of diethyl ether. The pH of the aqueous layer was then adjusted to 7.0, using 3 N H_2SO_4, and then reextracted 3 times with 80 mL of diethyl ether. The ether extracts were pooled, treated with anhydrous Na_2SO_4, and concentrated by rotary evaporation. The subsequent yellow oil was vacuum distilled to yield 98 mmol (35%) of 3-mercaptopropionitrile (colorless oil). The 3-mercaptopropionitrile had the following properties: bp 57 °C (6 mm); IR 2560 (SH) and 2240 cm^{-1} (CN); proton NMR δ 1.88 (SH, 1 H, s) and 2.68 (CH_2–CH_2, 4 H, q); ^{13}C NMR δ 20.48 (CH_2–CN, 1 C), 22.74 (CH_2–S, 1 C), and 118.33 (CN, 1 C); mass spectrum, *m/z* 87 (parent molecular ion).

Synthesis of 3-[(4-Azidophenyl)dithio]propionitrile. *N*-[(4-Azidophenyl)thio]phthalimide (25 mmol) was added to a solution of 3-mercaptopropionitrile (26 mmol) in 100 mL of benzene, refluxed for 10 h, and placed at −15 °C overnight in order to precipitate excess phthalimide. The reaction mixture was then filtered and the filtrate loaded onto a silicic acid column (5.5 × 45 cm) which was eluted with benzene, while 10-mL fractions were collected. Each fraction was then analyzed by TLC, using chloroform as the solvent, and the appropriate fractions were pooled and evaporated to dryness to give a yellow oil, with a yield of 14.2 mmol (57%). The compound isolated in this way had the following properties: R_f (chloroform) 0.6; IR 2260 (CN) and 2130 cm^{-1} (N_3); proton NMR δ 2.74 (CH_2CH_2, 4 H, q), 6.88 (aromatic, 2 H, d), and 7.42 (aromatic, 2 H, d); ^{13}C NMR δ 17.57 (CH_2–CN, 1 C), 33.43 (CH_2–S, 1 C), 119.89 (unsubstituted aromatic, 2 C), 130.42 (unsubstituted aromatic, 2 C), 132.79 (aromatic C–S, 1 C), 139.87 (aromatic C–N_3, 1 C), and 177.8 (CN, 1 C).

Synthesis of Methyl 3-[(4-Azidophenyl)dithio]propionimidate. This was synthesized according to the procedure of Ji (1977).

Derivatization and Iodination of Concanavalin A (Con A). Con A (Worthington Biochemical) (2 mg) was dissolved in 1 mL of 0.1 M Na_2HPO_4 (pH 8.5) containing 0.1 methyl α-D-mannoside, and to the solution, 5 μL of 200 mM NHS-APDP in acetonitrile was added every 15 min for a total of 60 min. The treated Con A was purified on a Sephadex G-25 column and radioiodinated as reported previously (Ji & Ji, 1980).

Results and Discussion

NHS-APDP and MADP. The schemes used to synthesize NHS-APDP and MADP are presented in Figures 1 and 2, respectively. Both schemes utilize *N*-[(4-azidophenyl)thio]-

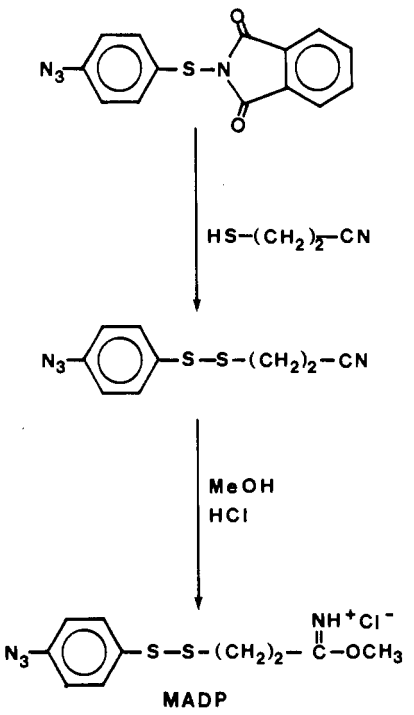

FIGURE 2: Scheme for the synthesis of MADP. The synthetic scheme begins with *N*-[(4-azidophenyl)thio]phthalimide, whose synthesis is outlined in Figure 1.

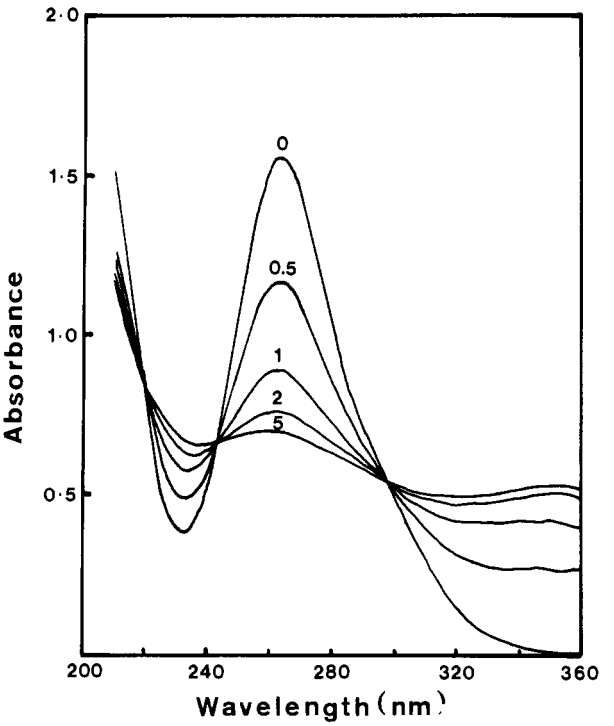

FIGURE 3: NHS-APDP (880 μM) in ethyl acetate was irradiated by placing the solution in a quartz cuvette 1 cm from a mineralight UVS-11 lamp. Following the indicated time periods (min), the ultraviolet spectra were recorded. It is known that photolysis of similar reagents is insignificant during the scanning (Ji, 1977).

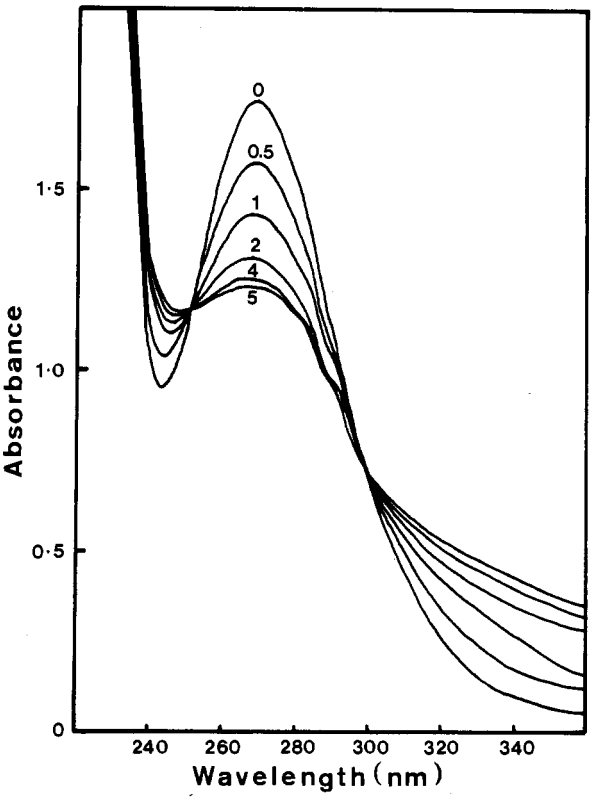

FIGURE 4: APDP–Con A was photolyzed as described in Figure 3.

phthalimide, in order to synthesize the mixed disulfides. The 4,4′-azidodiphenyl disulfide used in the reaction to form the phthalimide derivative was synthesized by using a two-step procedure, which involved the diazotization of the aromatic amine followed by treatment of the diazonium salt with sodium azide. *N*-[(4-Azidophenyl)thio]phthalimide was then synthesized from the 4,4′-azidodiphenyl disulfide by first chlorinating the disulfide after which the resulting sulfenyl chloride was reacted with phthalimide (Behforouz & Kerwood, 1968). The synthesis of the mixed disulfide involved refluxing *N*-[(4-azidophenyl)thio]phthalimide in the presence of the required sulfhydryl (Harpp et al., 1970). Although this procedure resulted in only moderate yields of the mixed disulfide, it should be considered as a general method for the synthesis of mixed disulfides, mainly because of its reliability and lack of appreciable amounts of side products. The *N*-hydroxysuccinimide ester of APDP was synthesized from the corresponding acid by using DCC as the coupling agent (Anderson et al., 1964), while the MADP was formed by treating the corresponding nitrile with hydrogen chloride in the presence of methanol (Pinner, 1892; Ji, 1977). The critical steps in the syntheses presented above are the synthesis of the *N*-[(4-azidophenyl)thio]phthalimide as well as the imidate and NHS ester, where anhydrous conditions are needed. All of the synthesized compounds, intermediate and final products, appeared as single spots on analytical silica gel TLC.

In addition to NHS-APDP, NHS-APDA, and MADP, another compound which also can be used as a cross-linking reagent is *N*-[(4-azidophenyl)thio]phthalimide. Both NHS esters and imidate react primarily with amino groups (Anderson et al., 1964; Cuatrecases & Parikh, 1972), while the thiophthalimide derivative could be used as a sulfhydryl-specific reagent.

Reaction of NHS-APDP with Con A. As expected, the absorption spectra of Con A changed as the lectin was coupled with NHS-APDP, due to the additional absorption by the reagent. When either the reagent or the Con A derivative was photolyzed, this increase in absorption due to the attachment of the APDP was lost (Figures 3 and 4). This decrease was then used to determine the stoichiometry of covalent attachment of APDP to Con A (Ji, 1977).

In order to use the APDP–Con A as a macromolecular affinity reagent, it is desirable to attach the maximum number of APDP moieties without affecting the biological activity of Con A. Since the NHS esters react primarily with free amines,

Table I: pH-Dependent Derivatization of Con A with APDP[a]

pH	APDP/Con A tetramer	pH	APDP/Con A tetramer
7.0	2.7	8.75	5.1
7.5	3.1	9.25	5.5
8.0	3.2	9.5	5.2
8.5	3.8	10.0	5.1

[a] Effect of pH upon the activation of Con A. Con A (1 mg) was dissolved in 0.5 mL of 0.1 M Na_2HPO_4 at different pHs adjusted with HCl. To this, 15 μL of 0.2 M NHS-APDP in dry acetonitrile was added every 15 min for a total of 60 min. The APDP–Con A was then separated from excess NHS-APDP by using Sephadex G-25 chromatography. The APDP–Con A was then subjected to photolysis and the number of the bound lectin derivative determined according to the formula previously described (Ji, 1977).

the attachment of the APDP moiety to Con A will be dependent on pH. To this end, we decided to determine the optimal pH for coupling the maximum number of APDP moieties to Con A. As summarized in Table I, the stoichiometry of attachment was found to increase with increasing pH, reaching a maximum at pH 9.25, after which it decreased. The increasing stoichiometry with increasing pH can be explained by the deprotonization of the amino group of lysine whereas the decrease above pH 9.25 was caused by the hydrolysis of NHS-APDP. When the pH optimum of 9.25 was used, it was found that NHS-APDP at a final concentration of 3 mM was sufficient to produce the maximum derivatization (data not shown). Before the binding activity of the ConA derivative was examined, the covalent attachment of the reagent was tested once more in an independent method. Since it is covalently coupled to Con A via an amide bond, reduction of the disulfide bond within the APDP moiety would release the phenyl azide group, and therefore the λ_{max} 263 nm would decrease. The choice of reducing agent is important, as such reagents are able to reduce azide groups to amino groups (Cartwright et al., 1976; Staros et al., 1978) and, in so doing, eliminate the absorption of the original azide. In this reduction, dithiols such as dithiothreitol were extremely efficient, whereas monothiols such as 2-mercaptoethanol and glutathione were much weaker (Staros et al., 1978). In the following test, the APDP–Con A was treated with 50 mM 2-mercaptoethanol for 60 min at room temperature; these conditions are known to cause very little if any reduction of azides (Staros et al., 1978). The reduced APDP–Con A showed a spectrum similar to that of native Con A, indicating that the phenyl azide group of the APDP moiety coupled to Con A can be completely removed by using a reducing reagent.

Binding Properties of the APDP–Con A. One of the major concerns in developing a macromolecular photoaffinity label is that the derivatization process may damage the binding activity of the macromolecule being modified. Therefore, the derivatization of Con A was carried out at pH 8.5 instead of 9.25 in order to decrease oligomer formation, which has been shown to increase with increasing pH (McKenzie et al., 1972). The second precautiion taken was the inclusion of 0.1 M methyl α-D-mannoside, as it was thought that the carbohydrate would protect its binding site against modification by NHS-APDP.

The APDP–Con A was tested for its ability to bind to human erythrocyte ghost membranes. Figure 5 demonstrates the binding of ^{125}I-labeled Con A to the ghost membrane. The binding increases rapidly up to a concentration of 100 nM, and then the Con A showed secondary binding characteristics. Results similar to these were obtained by Gordon & Young (1979), who suggested that Con A was exhibiting positive

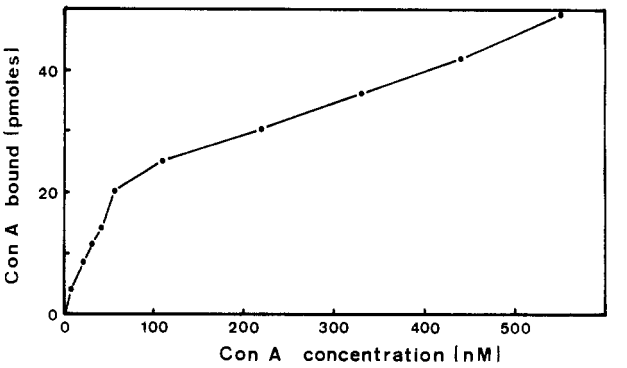

FIGURE 5: Concentration dependence of ^{125}I-labeled Con A binding to the human erythrocyte ghost membrane. The human erythrocyte ghost membrane was incubated in the presence of increasing concentrations of ^{125}I-labeled Con A, and the amount of ^{125}I-labeled Con A bound to the membrane was determined.

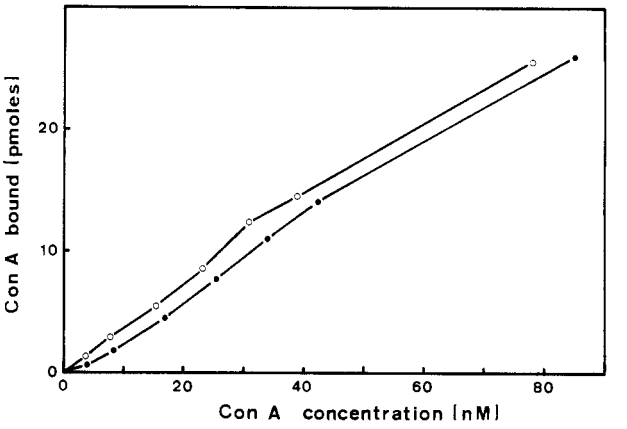

FIGURE 6: Binding of ^{125}I-labeled APDP–Con A to the human erythrocyte ghost membrane. Increasing concentrations of ^{125}I-labeled Con A (○) and ^{125}I-labeled APDP–Con A (●) were incubated with the membranes, and the amount of bound Con A was determined as described under Experimental Procedures.

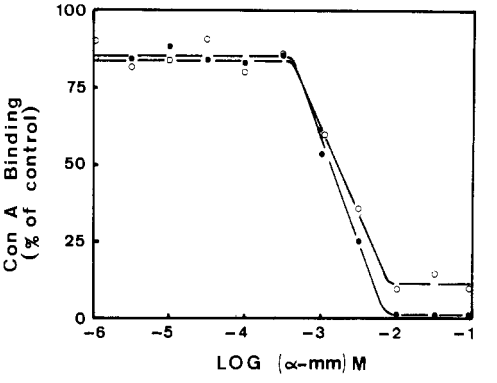

FIGURE 7: Inhibition of Con A binding by methyl α-D-mannoside. ^{125}I-Labeled Con A (○) or ^{125}I-labeled APDP–Con A (●) at a concentration of 100 nM was incubated with the human erythrocyte ghost membrane in the presence of increasing amounts of methyl α-D-mannoside, and the amount of Con A bound to the membrane was determined.

cooperativity in its binding to the human erythrocyte membrane. Similar results were also observed in Con A binding to rat thymocytes (Bornens et al., 1976), wheat germ agglutinin binding to CHO cells (Stanley & Carver, 1977) and to mouse teratoracarcinoma cells (Gachelin et al., 1976), and soybean agglutinin binding to human and rabbit erythrocytes (Reisner et al., 1976).

For determination of the specific, noncooperative interaction of Con A with its membrane receptor, Con A concentrations less than 100 nM were used. Both ^{125}I-labeled Con A and

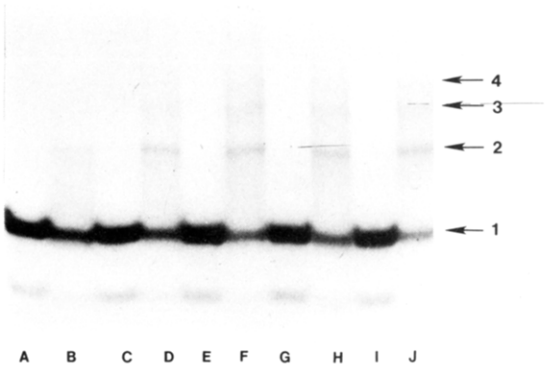

FIGURE 8: Polyacrylamide gel electrophoresis of ^{125}I-labeled Con A and ^{125}I-labeled APDP–Con A. Lanes A, C, E, and G are for ^{125}I-labeled Con A and the rest for ^{125}I-labeled APDP–Con A. Lanes A and B were not photolyzed. Lanes C and D were treated with one flash (Kiehm & Ji, 1977). Lanes E and F were photolyzed with a UVS-11 lamp for 2 min and Lanes G and H for 3 min. Lanes I and J were photolyzed for 4 min with the UV lamp, and then lane I was treated with 2-mercaptoethanol. The photolyzed samples were solubilized in sodium dodecyl sulfate and electrophoresed. The gel was overlaid with X-ray film.

^{125}I-labeled APDP–Con A bound to erythrocyte ghosts equally well (Figure 6), and the binding of both was inhibited by methyl α-D-mannoside to the same extent (Figure 7). The higher basal level observed for the ^{125}I-labeled APDP–Con A suggests that the Con A derivative has more nonspecific binding, but it is still less than 10% of the maximum binding.

It is concluded that the covalent attachment of APDP to Con A has little, if any, effect on the binding activity of Con A.

Cross-Linking of APDP–Con A. Con A is known to exist in dimers or tetramers, depending upon the pH (McKenzie et al., 1972). Therefore, the cross-links between subunits can be used as a test for the presence of the reagent in the lectin. Both ^{125}I-labeled Con A and ^{125}I-labeled APDP–Con A were photolyzed by using UV photolysis as well as flash photolysis (Kiehm & Ji, 1977), then treated with sodium dodecyl sulfate, and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 8). Prior to photolysis, the major band for both ^{125}I-labeled Con A and ^{125}I-labeled APDP–Con A corresponded to the monomer. The radioactive band migrating faster than the monomer is a proteolytic breakdown product of Con A, observed in most Con A preparations (Abe et al., 1971; Wang et al., 1971). Upon photolysis, no new band appeared for ^{125}I-labeled Con A. Yet, when ^{125}I-labeled APDP–Con A was photolyzed, bands corresponding to dimer and trimer increased, and a new band corresponding to tetramer appeared. The areas corresponding to the monomer, dimer, trimer, and tetramer were cut from the gel, and the radioactivity was determined. Before photolysis, 78% of the total radioactivity appeared in the monomer band; yet, after UV photolysis for 2 min, this was decreased to 34%, while there was a concomitant increase in the amount of radioactivity in the dimer, trimer, and tetramer bands (Table II). These oligomeric bands disappeared when the samples were treated with mercaptoethanol.

Table II: Photo-Cross-Linking of Con A[a]

	no UV[b]	UV[b]
monomer	78	34
dimer	18	21
trimer	3	13
tetramer	1	13

[a] Oligomer formulation upon irradiation of ^{125}I-labeled APDP–Con A. Following autoradiography, the bands corresponding to the monomers, dimers, trimers, tetramers, and higher oligomers were excised from the dehydrated gels (Figure 8, bands B and H) and the radioactivities counted. The counts in each oligomeric form are then expressed as a percentage of the total counts in all of the oligomeric forms. [b] Percentage of counts per minute.

Conclusion

We have synthesized and characterized three different photoactivable heterobifunctional cleavable reagents. One of them, NHS-APDP, was used to derivative Con A. A stoichiometry of attachment of up to 5.5 molecules of APDP per Con A tetramer was reached by measuring the absorption changes upon photolysis (Ji, 1977). The covalently attached phenyl azide moiety could be removed from APDP–Con A by treatment with 2-mercaptoethanol. The binding activity of APDP–Con A is similar to that of native Con A. It was also shown that photolysis of ^{125}I-labeled APDP–Con A resulted in the cross-linking of Con A monomers to give tetramer as the highest recognizable oligomeric form. These cross-links can readily be cleaved by reducing agents.

The cleavable cross-linking reagents can easily be attached to a variety of different macromolecules, thereby increasing the scope of macromolecular affinity labeling. Such affinity labeling has already been shown to be potentially useful (Ji, 1979), and the introduction of cleavable reagents should enable easier identification of the particular membrane receptor being studied.

References

Abe, J., Iwabuchi, M., & Ishii, S. I. (1971) *Biochem. Biophys. Res. Commun. 45*, 1271–1278.

Anderson, G. W., Zimmerman, J. E., & Callahan, F. M. (1964) *J. Am. Chem. Soc. 86*, 1839–1842.

Behforouz, M., & Kerwood, J. E. (1969) *J. Org. Chem. 34*, 51.

Bornens, M., Karsenti, E., & Avrameas, S. (1976) *Eur. J. Biochem. 65*, 61–69.

Cartwright, I. L., Hutchinson, D. W., & Armstrong, V. W. (1976) *Nucleic Acids Res. 3*, 2331–2339.

Cuatrecases, P., & Parikh, I. (1972) *Biochemistry 11*, 2291–2298.

Gachelin, G., Buc-Caron, M. H., Lis, H., & Sharon, N. (1976) *Biochim. Biophys. Acta 436*, 825–832.

Gordon, J. A., & Young, R. K. (1979) *J. Biol. Chem. 254*, 1932–1937.

Harpp, D. N., Ash, D. K., Back, T. G., Gleason, J. G., Orwig, B. A., Van Horn, W. F., & Snyder, J. P. (1970) *Tetrahedron Lett. 41*, 3551–3553.

Jaffe, C. L., Lis, H., & Sharon, N. (1980) *Biochemistry 19*, 4423–4429.

Ji, T. H. (1976) *Membranes and Neoplasia: New Approaches and Strategies*, pp 171–178, Alan R. Liss, Inc., New York.

Ji, T. H. (1977) *J. Biol. Chem. 252*, 1566–1570.

Ji, T. H. (1979) *Biochim. Biophys. Acta 559*, 39–69.

Ji, I., & Ji, T. H. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7167–7170.

Kiehm, D. J., & Ji, T. H. (1977) *J. Biol. Chem. 252*, 8524–8531.

McKenzie, G. H., Sawyer, W. H., & Nichols, L. W. (1972) *Biochim. Biophys. Acta 263*, 283–293.

Peters, K., & Richards, F. M. (1977) *Annu. Rev. Biochem. 46*, 523–552.

Pinner, A. (1892) *Die Imidoather Uno Ihre Derivate*, Oppenheim, Berlin.

Reisner, Y., Lis, H., & Sharon, N. (1976) *Exp. Cell Res. 97*, 445–448.

Stanley, P., & Carver, J. P. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5056–5059.
Staros, J. V., Bayley, H., Standring, D. N., & Knowles, J. R. (1978) *Biochem. Biophys. Res. Commun. 80*, 568–572.
Traut, R. R., Bollen, A., Sun, T., Hershey, J. W. B., Sunberg, J., & Pierce, L. R. (1973) *Biochemistry 12*, 3266–3273.
Wang, J. L., Cunningham, B. A., & Edelman, G. M. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1130–1134.

Aggregation-Linked Kinetic Heterogeneity in Bovine Cardiac Myosin Subfragment 1†

Duane P. Flamig‡ and Michael A. Cusanovich*

ABSTRACT: Studies of the cardiac myosin subfragment 1 concentration dependence of the rate constants for adenosine 5′-triphosphate (ATP) binding and steady-state hydrolysis reveal that the observed rate constants are remarkably dependent on the protein concentration. The kinetics for ATP binding are biphasic, and both the fast- and slow-phase rate constants and the respective fractions of fast and slow material vary as a function of protein concentration. Two different types of kinetic experiments were conducted, one in which the ATP concentration was fixed but the subfragment 1 concentration was varied and another for which the ATP/subfragment 1 ratio was fixed but both concentrations were varied. The results of these two experiments on cardiac subfragment 1 are consistent with an ATP-dependent reversible aggregation. Light-scattering experiments confirm the presence of this aggregation and the ATP dependence. Similar studies on rabbit skeletal subfragment 1 give monophasic, protein-independent kinetics consistent with a monomeric species in solution. A simple monomer–dimer mechanism can account for the cardiac subfragment 1 kinetic results when changes in tryptophan fluorescence are used. However, the light-scattering results show that cardiac myosin subfragment 1 undergoes multiple reversible molecular weight changes in solution and may be tetrameric at high concentrations.

The binding of nucleotides to bovine cardiac myosin and subfragment 1 has previously been shown to be biphasic (Taylor & Weeds, 1976). Preliminary studies in our laboratory on cardiac S1[1] have confirmed the biphasic kinetics and indicated that relative amounts of the two kinetic species as well as the corresponding rate constants were dependent on the S1 concentration. According to the proposed three-step mechanism for the binding and rapid hydrolysis of ATP by myosin

$$M + ATP \overset{1}{\rightleftharpoons} MATP \overset{2}{\rightleftharpoons} M^{**}ADP{\cdot}P_i$$

the observed rate constants should be independent of the concentration of myosin under pseudo-first-order conditions for ATP with respect to myosin. The simplest explanation for the observed biphasic kinetics of bovine cardiac S1 is that two protein species are present with each binding ATP with a different rate constant. However, this model predicts that the fractions of fast and slow material should be constant for all nucleotide concentrations, which is contrary to what is observed with cardiac S1. The next simplest model is to assume that there is protein–protein interaction, the simplest type being reversible aggregation. The fact that cardiac myosin has complex kinetics has limited studies on this form of myosin. In an attempt to resolve this problem in order to make cardiac myosin more amenable to detailed study, we have investigated the kinetic and aggregation properties of cardiac S1.

Initially, a series of experiments were designed in which the ATP concentration was held constant, and the protein concentration was varied and the apparent rate constant for ATP binding was measured. If the interconversion of the two forms of the protein is slow compared to nucleotide binding, then the fractions of fast and slow material will yield the monomer–aggregate equilibrium constant directly as well as the binding rate constants for at least two forms of the protein. The ATP concentration was chosen at an intermediate value (60 μM) in order to maximize the effect of S1 concentration on the observed biphasic binding reaction. Since it was observed that the fraction of fast material increased with increasing ATP concentration at a fixed protein concentration, a second set of experiments was carried out in which the ATP/S1 ratio was held constant by simultaneously varying both reactant concentrations. This experiment was designed to further discriminate between protein concentration dependent aggregation and ATP-induced dissociation. The observed increase in the contribution of the fast species with increasing ATP concentration suggests that the interconversion of the two or more forms of S1 is competitive with nucleotide binding to the active site. When the ATP/S1 ratio is kept small but still almost pseudo first order, the nucleotide-induced interconversion can be minimized.

Taylor & Weeds (1976) have shown from steady-state phosphate release experiments as a function of ATP concentration that there are at least two forms of bovine cardiac

† From the Department of Biochemistry, University of Arizona, Tucson, Arizona 85721. *Received January 19, 1981; revised manuscript received June 10, 1981.* This work was supported by National Institutes of Health Grant HL-20984 and by a Muscular Dystrophy Postdoctoral Fellowship to D.P.F.
‡ Present address: Department of Chemistry, University of Oregon, Eugene, OR 97403.

[1] Abbreviations used: S1, subfragment 1; ATP, adenosine 5′-triphosphate; ATPase, adenosine-5′-triphosphatase; BTP, bis(Tris)propane, 1,3-bis[[tris(hydroxymethyl)methyl]amino]propane; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; $NaDodSO_4$, sodium dodecyl sulfate; LC, light chain; PM, photomultiplier.

0006-2960/81/0420-6760$01.25/0