Binding of Chlorpromazine and Thioproperazine *in Vitro*

III. Fluorometric Measurement of Changes in *Limulus polyphemus* (Horseshoe Crab) Myosin B Structure and Enzyme Activity after Treatment with Phenothiazine Drugs

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SUMMARY

Chlorpromazine (CPZ) and thioproperazine (TPZ) were titrated fluorometrically with horseshoe crab myosin B. Resulting alterations in protein structure were indicated by discrete changes in the quenching and polarization of protein fluorescence, and in protein light scattering. These effects were demonstrated at drug concentrations from 0.23 to 13 μM with 46–263 μg of protein per milliliter. We observed cooperative effects of protein concentration upon the drug binding. At CPZ and TPZ concentrations less than 10 μM/mg of protein, drug binding was only 2.7–2.8 nmoles/mg of protein per milliliter. At drug concentrations above 100 μM/mg of protein, binding increased to 77–78 nmoles/mg of protein per milliliter. Equivalent minimal combining weights for the myosin B per mole of drug are 237,700 ± 2,000 g and 12,830 ± 710 g at drug concentrations below 10 μM and above 100 μM, respectively. Addition of 22 nmoles of TPZ per milligram of protein per milliliter to the ATPase assay medium increased enzymatic activity more than 100%. TPZ and trifluoperazine were also used as fluorescence microscopy stains for myosin B in intact sarcomeres. This procedure revealed the translocation of this protein within the lateral A band after contraction.

INTRODUCTION

Phenothiazine fluorescence permits spectrophotofluorometric studies of drug binding to various protein and subcellular fractions (1–3). Phenothiazine derivatives inhibit the activity of several enzymes, and these drugs affect the permeability of such lipoprotein membranes as mitochondrial and erythrocyte envelopes (4–6). This study reports on the binding of the phenothiazine drugs, chlorpromazine and thioproperazine, to a purified invertebrate contractile protein, myosin B, extracted from adult *Limulus polyphemus* (horseshoe crab). The reported localization of myosin B protein in the A band of *Limulus* sarcomeres has been confirmed (7, 8). Chlorpromazine and thioproperazine change the structure and ATPase activity of the purified protein. The binding of TPZ[2] and trifluoperazine to the sarcomeres of the muscle was also observed with fluorescent microscopy techniques.

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MATERIALS AND METHODS

General. Three methods were used to observe the effects of phenothiazines on *Limulus* myosin B at different levels of protein organization. First, fluorometry determined ligand concentrations at which binding to the purified soluble protein occurred. The fluorescence parameters observed were (a) quenching of the protein fluorescence by the phenothiazines, (b)

[2] The abbreviations used are: TPZ, thioproperazine; CPZ, chlorpromazine; TFPZ, trifluoperazine.

changes in fluorescence polarization (ρ_M) of the protein, (c) changes in light scattering of the protein, and (d) changes in fluorescence polarization of the drugs (ρ_D). Second, the ATPase activity of aggregated myosin B (9) was measured at different phenothiazine concentrations. Ligand concentrations were chosen for enzyme assay to correspond with those at which the drug induced marked changes in the fluorescence properties of the protein. Finally, fluorescent microscopy techniques (8) were used to examine the staining of homogenized, glycerinated *Limulus* skeletal muscle fibers by CPZ, TPZ, and TFPZ.

Preparation of the protein. Purified *L. polyphemus* myosin B, frozen at —23° in glycerol,[3] was precipitated by addition of equal volumes of water. The sediment that was obtained after centrifugation at 30,000 rpm for 60 min at 0° (rotor No. 50, Beckman model L-2 ultracentrifuge) was suspended and homogenized in 2.5–3 ml of 0.6 M KCl, pH 7.2. Unused fractions of this stock solution were stored at —23°. The different stock solutions contained 3.23–6.83 mg of protein per milliliter (10).

Drugs and other chemicals used. Chlorpromazine hydrochloride [2-chloro-10-(3-dimethylaminopropyl)phenothiazine hydrochloride, mol wt 355.33; Smith Kline and French], and thioproperazine ethanedisulfonate {dimethylsulfamido-3-[(methyl-4-piperazino)-3-propyl]-10-phenothiazine bismethanesulfonate, mol wt 638.84; Poulenc Ltd., Montreal} were diluted to 5 mg/ml with 0.6 M KCl, pH 7.2, for titrations. TPZ, 5 mg/ml in water, was used in the ATPase assay. TPZ and trifluoperazine {2-trifluoromethyl-10-[3-(methyl-4-piperazinyl)propyl]phenothiazine, mol wt 407.49; Smith Kline and French}, 5 mg/ml in water, were used as stains for fluorescence microscopy. The coenzymes AMP, ADP, and ATP (Sigma Chemical Company) (50 mM) were used for spectrophotofluorometric studies and ATPase assays. Distilled water was used in these experiments.

Spectrophotofluorometry. Fluorescent measurements and titrations were made with an Aminco-Bowman spectrophotofluorometer and were recorded with a Moseley 3S x-y recorder in parallel with a Photovolt Densicord recorder, as previously described (1). A 1% transmittance change caused a 2-inch recorder y-axis displacement, with signal to noise ratio of 26:1. The protein and drug solutions were placed in a 1-cm^2 quartz cuvette (final volume, less than 3 ml) in a thermostatically controlled cell compartment at 20° ± 0.2°. A Beckman/Spinco No. 151 microtitrator was used to deliver nanoliter volumes of drug through a stirrer shaft mounted in the cell compartment cover (3).

The protein and nucleotides were titrated in 0.6 M KCl, pH 7.2. After each addition of drug, three to seven protein fluorescence spectra were recorded. The apparent partial polarization of fluorescence (ρ) was calculated by standard methods (11). Light scattering measurements were made by recording emission at a 90° angle to the excitation path at the 285 mμ excitation wavelength, simultaneously with the measurement of the fluorescence at 350 mμ.

To analyze the data from the titrations, a provisional assumption was made that the amount of initial quenching at the highest protein concentration (263 μg/ml) by the smallest drug addition (7.4×10^{-10} mole of TPZ) indicated the effect of *complete binding*. Thus, the data were transformed to those shown in Fig. 1b. The amount of bound drug, r, was determined from the smallest concentration of drug per milligram of protein, D', which caused the maximal initial quenching of the protein fluorescence at the start of the titration with the greatest concentration of protein that was practical.

ATPase activity. The procedure of de Villafranca and Naumann (9) was used. Assay conditions were maintained that were reported to be optimal for enzymatic activity, i.e., less than 230 mM KCl and 50 mM Ca^{++}, at pH 9.0 (9). TPZ and myosin B solutions were prepared containing 8.6, 11.0, 17.3, and 22.1 μM TPZ per milligram of protein and assayed for ATPase activity. These ligand concentrations are

[3] Supplied by Dr. G. W. de Villafranca of Smith College, Northampton, Massachusetts.

within the range of the fluorometric titration procedures. In the phosphate determinations, tubes containing TPZ were corrected for a small color increment due to TPZ alone.

Fluorescence microscopy and photomicrography. Glycerinated *Limulus* muscle fibers were teased by brief homogenization in a solution of 25% glycerol and 75% 6.7 mM KH_2PO_4 containing 40 mM KCl (pH 7.2), and were washed by centrifugation at 800 *g* for 10 min with the same buffer (12). They were stained with 25 μg of either TPZ or TFPZ in 5 μl of buffer, and were observed through a Zeiss WL fluorescence microscope. The drug fluorescence was activated through the Zeiss excitation filter III and a Photovolt filter with maximal transmission at 365 mμ. Light was collimated with a condenser, "auxiliary lens II," and was viewed with barrier filters transmitting at 500 mμ.[4] Photographs were made with Kodachrome II and Ektachrome films using 16×, 40×, and 100× oil-immersion objectives.

RESULTS

The polarization of fluorescence (ρ_M) of 46–263 μg of myosin per milliliter was $+0.253 \pm 0.002$ at 20° in water, 0.9% NaCl, and 0.6 M KCl. The uncorrected fluorescence maximum appeared at 348 mμ when activated at 285 mμ. The fluorescence increase of myosin B was linear with additions of protein from 0 to 147 μg/ml in H_2O or 0 to 105.4 μg/ml in KCl in a 1-cm² cuvette. No changes in protein or drug fluorescence characteristics were observed in blank titrations, in which each ligand was exposed separately under identical conditions of illumination, mixing, and temperature for lengths of time equivalent to a drug-binding titration.

Effects of TPZ and CPZ on Myosin B as Measured by Spectrophotofluorometry

Quenching of protein fluorescence. Results from titrations with TPZ are used for illustrative purposes. More extensive analysis was performed with this compound because the fluorescence of TPZ (523 mμ) and that of partially oxidized TPZ (440 mμ) are completely separated from the light scatter (285 mμ) and fluorescence (350 mμ) of the protein (1, 13).

Minute additions of TPZ (only 10^{-7} M) quenched protein fluorescence (Fig. 1a). However, at 7.9 μM TPZ per milligram of protein, a sharp increase in protein fluorescence occurred if less than 200 μg of protein per milliliter was present. This occurred in both water and 0.6 M KCl titration media. The increase in emission at 350 mμ was sometimes greater than the fluorescence of native myosin B. At a concentration of 263 μg of protein per milliliter, a similar increase in fluorescence was seen in the titration at 17 μM TPZ per milligram of protein. Further additions of TPZ, past the drug-induced fluorescence peak, caused additional quenching, as shown in Fig. 1a, curve labeled Q_2. At all protein concentrations, quenching of the fluorescence reached a minimum at 21–34 μM TPZ per milligram of protein. Further decreases in emission at 350 mμ were due to inner filter effects of TPZ. In addition, protein concentrations above 270 μg/ml produced sufficient inner filter effects to interfere with measurement of quenching.

Two sets of points were obtained from titrations with both the high and low protein concentrations. The initial set, labeled m_1 and m_2 in Fig. 1b, have identical slopes, differing only in position because of the apparently incomplete drug binding at the start of the titrations with low protein concentrations. Initially, with protein concentrations less than 200 μg/ml, $r/D = \frac{1}{3}$ of the value obtained with protein concentrations above 200 μg/ml. However, after the sharp increase in fluorescence of the protein, additional binding was different, within both protein concentration ranges, from that at the start of the titration. With 214–263 μg of protein per milliliter, the secondary fluorescence quenching was greater per microgram of TPZ than at the start of the titration. In comparison, at lower protein concentrations (46–200 μg/

[4] We thank Professor H. I. Hirshfield, Biology Department, Graduate School of Arts and Sciences, New York University, for permission to use the fluorescence microscopy equipment.

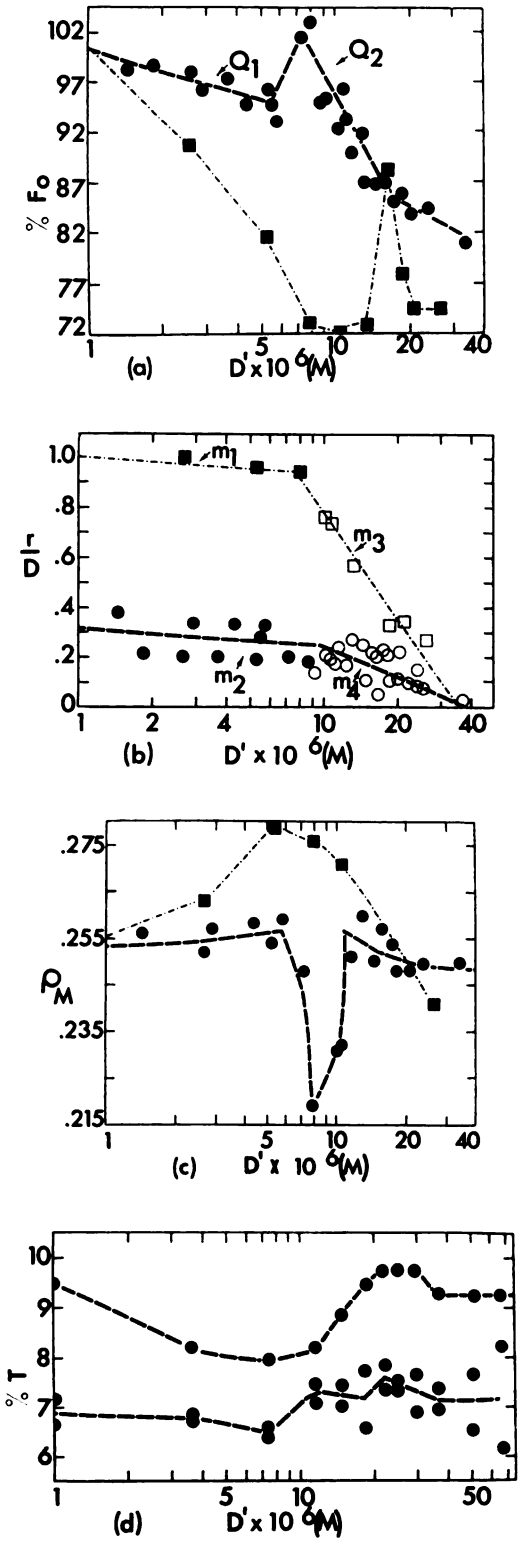

FIG. 1. *Effect of thioproperazine on the fluorescence emission, polarization, and light scattering of* Limulus *myosin B*

a. Quenching of protein fluorescence by TPZ. The ordinate is the percentage of initial protein fluorescence (% F_0, activated at 285 mμ, observed at 350 mμ) corrected for dilution. Each point is the average of three to seven spectra, identified as follows: ●, mean fluorescence of seven titrations with protein concentrations of 46–200 μg/ml; ■, mean of three titrations with 234–263 μg/ml. All these titrations were performed with the electrical vector of the analyzing polarizer perpendicular to the excitation polarizer. D' is the molar concentration of drug per milligram of protein.

b. Transformation of fluorescence quenching data to the r/D form to demonstrate differences in binding at two protein concentrations (14). Symbols are identified in Fig. 1a; D is the molar drug concentration. The open symbols are the corresponding means of fluorescence emission intensity of the protein after the first change in slope of the titration curves shown in Fig. 1a.

c. Changes in the polarization of protein fluorescence (ρ_M) caused by TPZ. All emission intensities were corrected for dilution by the titrating fluid. ●, Averages of four titrations with protein concentrations between 80 and 200 μg/ml; ■, averages of two titrations with 263 μg of myosin B per milliliter.

d. Measurement of light scattering changes of myosin B after addition of TPZ. The analyzing and excitation polarizers were set with electrical vectors vertical, in parallel to the lamp axis and diffraction grating blaze. Excitation and fluorescence were adjusted at 285–290 mμ to produce the greatest reading on the meter and recorder. Other conditions were identical with those for Fig. 1a and c. The upper curve, beginning at 9.5% transmittance (T), is a plot of the results from the titrations with 263 μg/ml of protein solution. The lower curve is the average of results from two titrations with 80 and 184 μg/ml of protein solutions. Changes in light scattering at the absorption wavelength do not account for the changes in fluorescence parameters. The cell compartment optical system contained two 1/16-inch slits in the excitation light path and a 1/8- and a 3/16-inch slit on the cuvette and grating sides of the emission polarizer prism, respectively. The exciting wavelength was 285 ± 1 mμ. The bandwidth of the excitation peak was recorded as −3, +6 mμ at 70% of the emission intensity. The bandwidth of the fluorescence was −20, +25 mμ under the same conditions, at 11×10^{-6} M TPZ per milligram of myosin B (263 μg of protein per milliliter), where the absolute and relative change in intensity were the greatest for all the titrations.

ml), the secondary change was much less (Fig. 1a, Q_1 vs. Q_2; Fig. 1b, m_2 vs. m_4).

Polarization of protein fluorescence. The first sharp change in polarization of protein fluorescence occurred at 5.3 μM TPZ per milligram of protein with 80–263 μg of myosin B per milliliter. The polarization of protein fluorescence at 350 mμ, at the lower myosin B concentrations, reached an average of +0.257 at 5.3 μM TPZ per milligram of protein and decreased to +0.219 at 8.0 μM TPZ per milligram of protein. A second polarization increase appeared at 14 μM TPZ per milligram of protein. Thereafter the polarization of protein fluorescence decreased slowly. No changes in this parameter occurred after 20 μM drug per milligram of protein was present (Fig. 1c). At the highest protein concentration that we used, the polarization changes were marked, but less discrete. At 263 μg of protein per milliliter, the change in polarization of protein fluorescence increased from +0.255 to +0.278 at 5.3 μM TPZ per milligram of protein and then decreased to +0.240 ± 0.003 at 27–60 μM TPZ per milligram of protein.

Changes in light scattering by myosin B. Light scattering at 285 mμ decreased at the same drug concentration that caused the first fluorescence peak with 80–200 μg of protein per milliliter. The scattering increased thereafter (Fig. 1d). Changes in the light scatter of the high-concentration protein solution were more marked and delayed than in the other solutions.

Polarization of drug fluorescence. The increase in polarization of fluorescence of the drugs at 465 mμ (CPZ) and 525 mμ (TPZ) was linearly related to protein concentration, and indicated the binding of both drugs (Fig. 2). Protein concentrations at which binding was theoretically complete were determined by the intersection of the slope described by the data points for initial polarization of drug fluorescence (ρ_D) with the lower limit of the fluorescence polarization of the concentrated protein (+0.243) (11, 13). The drug to protein binding ratio was calculated in terms of the minimum molecular weight of the protein that could bind 1 molecule of drug, from data including those shown in Fig. 2.

There is no significant difference between the affinities of CPZ and TPZ binding to myosin B measured by polarization of drug fluorescence, if the initial concentration of either phenothiazine is less than 10 μM/mg of protein. In this case, the binding of 1 mole of TPZ or CPZ indicates that an equivalent weight of *Limulus* myosin B is 237,700 ± 2,000 g of protein. In contrast, if the protein is added to relatively concentrated drug (more than 0.1 mM/mg of protein), the binding equivalence is 12,830 ± 710 g of protein per mole of

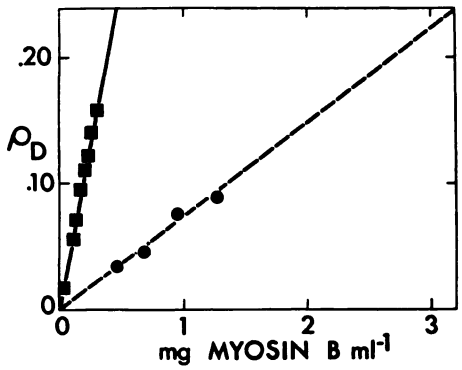

FIG. 2. *Increase in polarization of fluorescence (ρD) of TPZ and CPZ during titrations with* Limulus *myosin B.*

The symbol (■) represents polarization of fluorescence of CPZ (13.85 μg/ml, 39 μM, in 0.6 M KCl, pH 7.2), activated at 340 mμ and read at 465 mμ. Protein was added in 10 μl amounts, containing 32 μg. The initial CPZ concentration was 1.22 mM/mg protein. The symbol (●) represents polarization of fluorescence of TPZ (2.94 μg/ml, 4.6 μM, in 0.6 M KCl, pH 7.2), activated at 365 nm and read at 525 nm. Protein was added in aliquots of 10 μl, containing 68.4 μg. The initial TPZ concentration was 67.2 μM/mg protein. Intermediate data points falling exactly on the titration curves are omitted for the sake of clarity. The maximum concentration of myosin B used was 1.3 mg/ml. In both cases the drug's fluorescence was measured with the electrical vector of the analyzing polarizer both parallel and perpendicular to the vertical electrical vector of the excitation polarizer. The graphs are terminated at $\rho D = +0.243$, corresponding to the minimum ρ_M observed for concentrated protein. There is an apparently greater affinity of the protein for the phenothiazine when the initial drug concentration is high (■).

either phenothiazine. Calculations from the slope of the polarization of drug fluorescence titrations indicate that 2.7 nmoles of TPZ and 2.8 nmoles of CPZ may bind to 1 mg of protein. However, if the protein is relatively dilute in comparison to the drug, i.e., if the drug concentration is 0.1 mM/mg of protein, the binding may be as high as 78 nmoles of CPZ and 77 nmoles of TPZ per milligram of protein. We therefore calculate that there are at least 18 ± 1 binding sites available for the phenothiazine drugs when $D' > 10^{-4}$ M for each site available at $D' < 10^{-5}$ M.

Evidence that CPZ may not be bound in the same way as TPZ was obtained from quenching titrations. The first increase in protein fluorescence in the CPZ titrations was observed at 1.7×10^{-5} M CPZ per milligram of protein when 62.7 μg of myosin B were present in 0.6 M KCl. Additional CPZ caused further quenching as in the TPZ titrations. However *at this low protein concentration,* the relative amount of CPZ causing the increase in protein fluorescence was equal to the TPZ concentration that caused the same effect at the *high* protein concentration. In addition, changes in ρ_M at 46–124 μg of protein per milliliter by CPZ were similar to those resulting from TPZ at the 263-μg/ml concentration.

In contrast to the results obtained with CPZ and TPZ, titrations of ATP, ADP, and AMP with myosin B in 0.6 M KCl showed no quenching or polarization change of the protein fluorescence after stepwise addition of these phosphonucleotides in 0.6 M KCl.

ATPase Activity

TPZ concentrations per milligram of protein equivalent to those occurring after

TABLE 1

Effect of thioproperazine on the ATPase activity of freshly prepared and aged Limulus *myosin B*

Limulus myosin B and drug were mixed in quadruplicate assay tubes in volumes of 0.1 ml, as follows: 0.055 or 0.060 ml of 0.6 M KCl (pH 7.2) containing 376.4 or 294.0 μg of protein with 8.6–22 μmoles of TPZ per milligram of protein in 0.045 or 0.040 ml of water, respectively. The other tubes received equivalent volumes of KCl and water. The final volume in each tube was 1.90 ml, and contained 0.1 mM histidine buffer, pH 9.0, 10 μM $CaCl_2$, and water, added in that order. The tube contents were mixed after each addition. The reaction was started by the addition of 0.1 ml of 0.05 M ATP (or water). Enzymatic activity in each pair of tubes was stopped at 0 or 10 min by the addition of 0.5 ml of 20% (w/v) trichloracetic acid. After colorimetric determination of inorganic phosphate (P_i), the results for each pair of tubes were averaged. In tubes marked "F" freshly thawed myosin B was present at a final concentration of 117.6 μg/ml. In tubes marked "A," aged myosin B, stored for 8 days at 4°, was present at a final concentration of 150.56 μg/ml.

Additions to incubation medium			P_i produced after incubation for		Relative enzymatic activity after 10 min[a]
Myosin B	ATP	TPZ	0 min	10 min	
		nmoles/mg protein	*nmoles/mg protein/ml*		
	+		15.9[b]	18[b]	
F	−		52.8	45.7	
F	+		194.7	658.8	100
F	+	11.0	218.5	843.2	137
F	+	22.1	206.6	1,225.7	219
	+		18.7[b]	17.5[b]	
A	−		6.4	7.8	
A	+		112.9	213.8	24
A	+	8.6	126.2	221.2	23
A	+	17.3	132.2	267.7	36

[a] Enzymatic activity was obtained by subtracting the sum of P_i produced in media containing ATP only or myosin B only from the amount of P_i released when myosin B and ATP were present in the incubation medium without TPZ.

[b] Concentration in nanomoles per milliliter (no protein present).

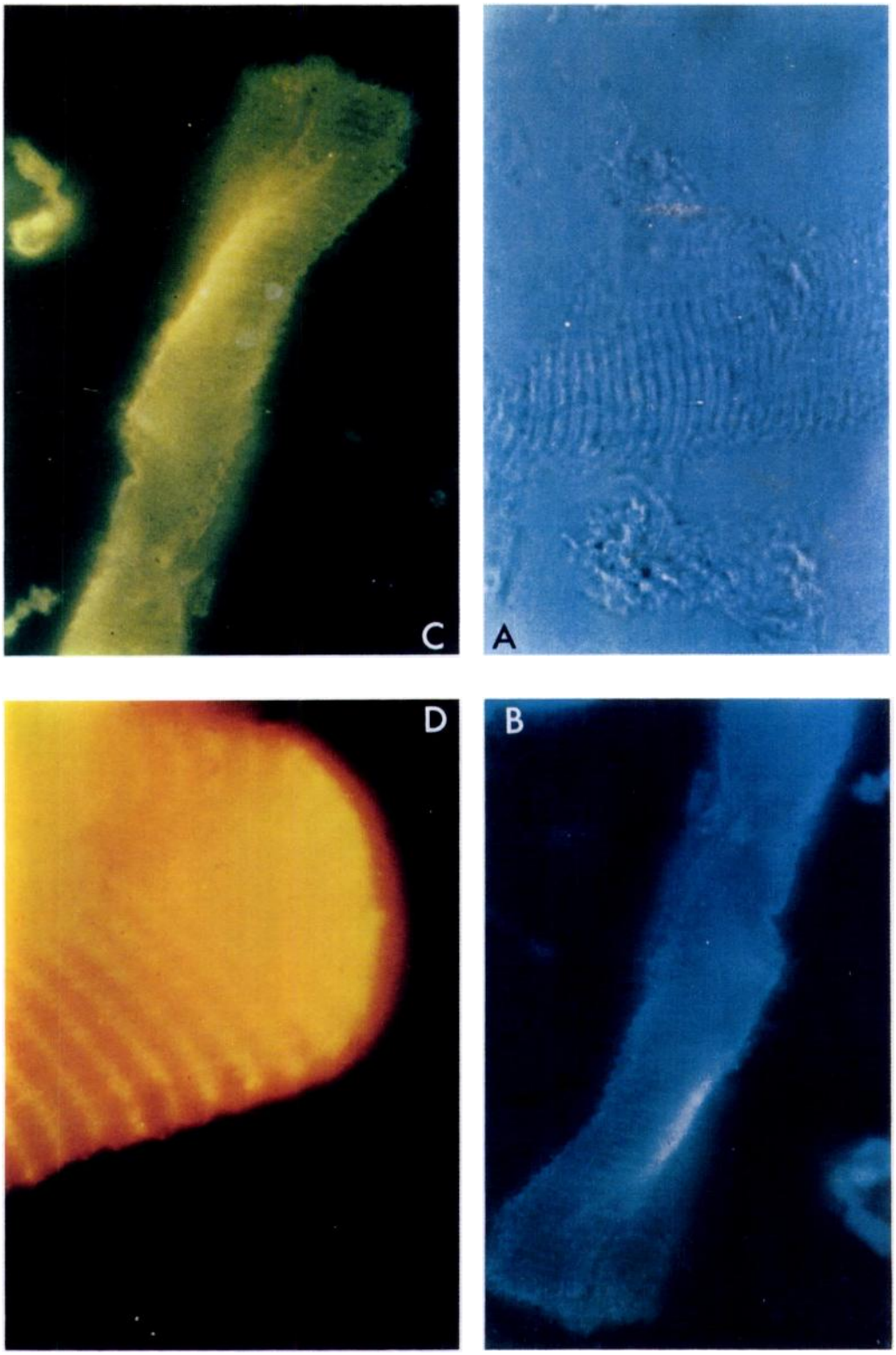

FIG. 3

the initial changes in the fluorescence titrations caused a significant increase in myosin B ATPase activity above that of the native protein (Table 1). TPZ alone had no effect on the phosphate released from ATP with or without heat-denatured enzyme (Table 2). There was an increase in ATPase activity of myosin B above the activity produced by Ca^{++} in the presence of TPZ concentrations per milligram of protein equivalent to, or greater than, that producing slopes m_3 and m_4 in the fluorescent quenching titrations. TPZ above 10^{-4} M inhibited the ATPase activity.

TABLE 2

Effect of thioproperazine on the ATPase assay

Assay conditions were identical with those given for Table 1, except that freshly thawed myosin B was heated at 80–100° for 15 min to produce the heat-inactivated myosin B (final concentration, 150.56 μg/ml) that was present in tubes marked "H."

Additions to incubation medium			P_i produced after incubation for	
Myosin B	ATP	TPZ	0 min	10 min
		nmoles/ml	*nmoles/ml*	
	+		11.9	11.9
H	−		14.7[a]	6.3[a]
	−	1.3	1.4	1.3
	−	2.6	2.0	1.4
H	+		87.4[a]	71.3[a]
	+	1.3	15.2	12.3
	+	2.6	13.2	11.8
H	+	8.6[a]	81.8[a]	93.7[a]
H	+	17.3[a]	96.5[a]	99.9[a]

[a] Concentration in nanomoles per milligram of protein per milliliter.

Fluorescence Microscopy

Without drug, the muscle fiber fragments appeared as schlieren patterns on a green background (Fig. 3a). TPZ fluorescence, an intense yellow-green, was observed first on the surface of the fibers (Fig. 3b). Within 3–5 min the TPZ fluorescence was symmetrically distributed in the lateral A band regions of the slightly contracted sarcomeres. Further shortening of the fibers and sarcomeres, to the "supercontracted" configuration (8), invariably followed TPZ administration (Fig. 3c).

Exposure to the drug for 5–10 min caused disruption of the fibrils. Disintegration began at the surface, which became intensely fluorescent. This was followed by disorganization of the internal banding of the fibrils, which yielded an amorphous, fluorescent precipitate. When TFPZ was used as a stain, fluorescence was not apparent immediately, but developed after exposure to the activating ultraviolet light. Thereafter, localization of TFPZ fluorescence, contraction, and subsequent disintegration of the fiber fragments proceeded similarly to TPZ (Fig. 3d).

DISCUSSION

Fluorometry of Drug Binding

Quenching of protein fluorescence by the phenothiazine drugs is, by itself, only qualitative evidence of drug binding because the drug is usually present in excess of the amount that may actually be bound. The 2.7 nmoles of TPZ necessary to bind to 1 mg of myosin B per milliliter (Fig. 2) were present early in the quenching titrations. The relative affinity of the dilute

FIG. 3. *Photographs of teased* L. polyphemus *muscle stained with TPZ and TFPZ using fluorescence emission from the bound drugs*

The filter arrangements are described in MATERIALS AND METHODS. The film colors in photographs b–d are not those that are perceived visually (see RESULTS).

a. Teased fibril as *seen* before addition of drug to slide; 40× objective, Kodachrome II, 2-min exposure.

b. Two minutes after 5 μl, of TPZ (5 mg/ml) were added to the slide surface, a 1.5-min exposure was taken of the same subject.

c. Two minutes later, after photograph b was taken, the fluorescence was more intense; 1.5-min exposure.

d. This photograph of another muscle preparation was taken on Ektachrome film, using immersion oil and a 100× objective. The exposure duration was 3 min; 10 min after 5 μl of TFPZ (5 mg/ml) were applied to the slide. The fibril appeared bright blue on a very dark red background.

protein for the drug is qualitatively indicated by continued quenching of the protein fluorescence after more TPZ was added.

However, the polarization of the drug fluorescence is zero in the absence of protein. The increase in polarization of drug fluorescence (ρ_D) (Fig. 2) with additions of myosin B is due to protein binding. The proportion of the drug molecules bound to the protein is $r = \rho_D/\rho_{protein}$. A value of 1 for r occurs when all the drug is protein-bound. The premise for this assumption is that drug molecules, which rotate randomly prior to emission after excitation (the rotational relaxation time is less than the mean lifetime of the excited state), become restricted in movement when protein-bound. Therefore, the observed ρ_D is a function of the number of drug molecules having the rotational relaxation time of the protein in the total drug molecule population. This assumption that $\rho_D = kr$ is valid when the fluorescence activity of the drug is not affected by binding. Under the measurement conditions used in this laboratory, phenothiazine fluorescence can be determined in the presence of protein (1–3). Continuously recorded spectra of the unoxidized phenothiazine derivatives show no emission intensity changes upon protein binding (3, 13).

Our results indicate that 7.5 μM TPZ per milligram of protein produces more than one effect on myosin B, depending upon the initial protein concentration. At a protein concentration of less than 200 μg/ml, or when the protein is soluble (e.g., in 0.6 M KCl), conformational changes in the protein apparently produced more binding sites, because additional quenching occurred when more drug was added (Fig. 1b, m_4), whereas the polarization of protein fluorescence at myosin B concentrations below 200 μg/ml was stabilized. More evidence was obtained from the mixtures of low concentrations of protein with a relatively high drug concentration. These solutions showed a change in the polarization of the drug fluorescence that indicated a new and very high affinity of the protein for the drug, 77–78 nmoles of drug per milligram of protein per milliliter. In contrast, with low phenothiazine concentrations (less than 10^{-4} M/mg of protein), the apparent drug binding was only 2.7–2.8 nmoles/mg of protein per milliliter.

Unfolding of the dilute protein would most readily make more fluorophore sites measurable, increase the quenching per nanomole of drug, and produce a transient decrease in polarization of protein fluorescence. This alteration might be accomplished by the extroversion of normally interiorly positioned fluorophore amino acid residues, transiently producing either a more spherical or a less rigid macromolecule.

Addition of TPZ to a concentrated (263 μg/ml) protein solution may also have produced aggregation of myosin B, shown by the increase in polarization of the protein fluorescence and the increased quenching (Fig. 1). The slope of m_3 was 3 times that of m_4 (Fig. 1b). Therefore, secondary quenching appears dependent upon protein, as well as drug, concentration, and represents a different type of binding from that plotted as m_1 and m_2. Possibly cooperative binding effects by drug and protein occurred under these conditions. This has been observed with these drugs in binding studies with mitochondria (1, 2, 13).

In comparison with the results from the phenothiazine titrations, the absence of fluorometric evidence for nucleotide binding to myosin B is inconclusive, since at this ionic strength and pH myosin B is soluble. This is not the optimal ionic strength or pH for ATPase activity of the protein (9), nor was Ca^{++}—reported to be necessary for ATP binding to the protein (15)—added to the cuvette.

ATPase Activity

The increase in ATPase activity of *Limulus* myosin B in association with TPZ contrasts with previous reports of membranal ATPase inhibition with the phenothiazines (5). Altered membranal permeability, also reported as an effect of phenothiazine binding (6), together with enzyme inhibition, may be due to the strong electron acceptor activity of these drugs (1, 5) as they compete with normal

coenzymes for energy transfer at lipoprotein surfaces. However, the myosin B ATPase reaction does not involve the same mechanisms. Part of the observed stimulating effect of TPZ on enzymatic activity may be due to the drug-initiated aggregation of the protein molecules in the concentrated protein solutions used for the assay. Aggregation of myosin B macromolecules appears to be necessary for ATP hydrolysis, because little enzyme activity is observed under conditions in which the protein is most soluble. Indeed, "optimal" conditions for ATPase assay—suspension of myosin B in basic medium (pH 9.0) of low ionic strength (less than 0.23 M KCl), and the presence of Ca^{++}—yield a particulate, insoluble aggregate of the protein. Under these conditions, protein-protein interactions may provide surfaces available for ATP binding, and TPZ treatment may have enhanced the probability of these interactions by altering the protein structure.

The polarization of fluorescence of myosin B (+0.255) is typical of an asymmetrical molecule. Vertebrate myosin A has been described as an elongated rod (16). Myosin B has enzymatic properties similar to those of vertebrate myosin A (9), and is localized with the A band of glycerinated *Limulus* sarcomeres (7, 8, 10) as myosin A is in glycerinated vertebrate sarcomeres (17). A molecular weight of 261,000 was proposed for the heavy meromyosin moiety of rabbit myosin A prepared in 5 M guanidine (18), and heavy meromyosin contains the ATPase site of the myosin A molecule (19). Therefore, if *Limulus* myosin B is structurally similar to vertebrate myosin A, both the effect of TPZ on the ATPase activity of the protein and the minimum equivalent weight that we calculated for the binding of 1 mole of TPZ (235.7–239.7 $\times 10^3$ g of protein) suggest that only 1 TPZ molecule can be bound without structural change.

Fluorescence Microscopy with Phenothiazines as Stains

Staining of teased, washed, glycerinated *Limulus* myofibrils with the phenothiazines permitted observation of drug effects on the protein in a higher organizational state, as it occurs in muscle tissue. The localization of TPZ within the A bands confirms the report, based on fluorescent antibody studies, of the lateral translocation of myosin B within the A band during contraction (8). Continued shortening as well as change in the banded appearance of the sarcomeres to the "supercontracted" state (8) is a direct visualization analogous to fluorometric observations of changes in emission and polarization of fluorescence of the concentrated purified protein after TPZ addition. The amount of drug applied to the slides (about 25 μg) was much larger than that in the protein solutions in the fluorometer cuvette, in comparison to the amount of fluid volume present. This *high drug concentration* undoubtedly was responsible for the ultimate disintegration of the bands, sarcomeres, and myofibrillar organization. The specificity of the staining of myofibrils by the phenothiazine appears to be identical with that of the fluorescent antibody for the protein, with considerably less background fluorescence. A drawback to the use of the drugs as stains, however, is their disruptive effects on the muscle preparations after exposure to ultraviolet light. *This cannot be interpreted as a toxic phenomenon.*

The conformational change of myosin B upon TPZ binding is of further interest, because the usual myosin conformational changes observed during contraction, such as decrease in the size of the A band and translocation of fluorescent antibody-stained myosin within the A band of *Limulus* sarcomeres (8), are probably the result of Ca^{++} and ATP binding (15) to the protein molecular aggregate. Identical structural modifications of the contractile protein have been observed with ultraviolet microscopy in the presence of TPZ and TFPZ without addition of ATP or Ca^{++} to the glycerinated muscle fibrils. Thus, the drug-induced configurational changes, and attendant sarcomeric contraction, may be analogous to contractile events previously observed with the glycerinated muscle models (8).

The production of configurational changes in proteins by these neuroleptic drugs which affect enzymatic activity may offer an explanation of their activity *in vivo*.

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