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Human α-Thrombin Binding to Nonpolymerized Fibrin–Sepharose: Evidence for an Anionic Binding Region†

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ABSTRACT: In order to investigate ligand binding sites in α-thrombin that interact with nonpolymerized fibrin, fibrinogen was conjugated (with CNBr) to Sepharose 4B and converted to the nonpolymerized fibrin resin with α-thrombin. Human α-thrombin was bound to the resin at 22 °C and eluted with a linear NaCl gradient [50–300 mM in 50 mM tris(hydroxymethyl)aminomethane hydrochloride, pH 7.6] with midpeak elution occurring at an ionic strength that corresponds to 170 ± 5 mM NaCl. Among various ligands examined, ATP and its analogues caused α-thrombin to elute with 125 mM or less salt. Apparent dissociation constants were estimated by the dependence of elution volume on ligand concentration. The most potent ligands for desorption from the column were anionic (e.g., adenine nucleotides), which also inhibit thrombin esterolytic/amidolytic and clotting activity [Conery, B. G., & Berliner, L. J. (1983) *Biochemistry 22*, 369–375]. The desorption series was at 10 mM concentrations: ATP = ADP > pyrophosphate > citrate > oxalate > PO_4^{3-}. Contrastingly, serotonin and related apolar compounds did not cause dissociation of α-thrombin from the fibrin resin, even though several of these substances inhibit fibrinogen clotting and esterolytic/amidolytic activities of the enzyme. These data imply that independent sites for apolar and anionic binding in α-thrombin are required for converting fibrinogen into clottable fibrin and that α-thrombin–fibrin binding involves an anionic site.

Thrombin has central bioregulatory functions in thrombosis and hemostasis. Upon generation during vascular injury, procoagulant α-thrombin interacts with platelets, endothelial cells, fibrinogen, and other components of the coagulation system (factors V, VIII, and XIII, protein C, etc.) to cause the formation of a hemostatic plug (Seegers, 1977; Fenton, 1981).

The conversion of fibrinogen by α-thrombin into clottable fibrin monomers occurs first at the Arg-16–Gly-17 bond in the Aα chains and subsequently at the Arg-14–Gly-15 bond in the Bβ chains with the release of fibrinopeptides A and B, respectively (Blombäck et al., 1978). Whereas the fibrinopeptides are small and formed as the second products of enzymic catalysis, the fibrin monomer is large and constitutes the first product of peptide bond cleavage. The monomer subsequently polymerizes to form fibrin strands, where polymerization is generally the rate-limiting process of clotting

†Supported in part by USPHS Grants HL 24549 (L.J.B.) and HL 13160 (J.W.F.) from the National Heart, Lung and Blood Institute. A preliminary report of this work was presented at the Xth International Society of Thrombosis and Hemostasis Congress, San Diego, CA, July 1985.
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0006-2960/85/0424-7005$01.50/0

(Higgins et al., 1983). During polymerization, α-thrombin (or its catalytically inactivated forms in the presence of sufficient active enzyme to promote a clot) incorporate into the clot (Lui et al., 1979). On the other hand, neither nonclotting human γ-thrombin nor the hirudin complex of α-thrombin incorporate into clots, suggesting the involvement of an unique binding site in α-thrombin (Wilner et al., 1981).

By conjugating fibrinogen to CNBr-activated agarose and subsequently exposing it to α-thrombin, the nonpolymerized fibrin resin is formed (Heene & Matthias, 1973). Such resins (at near neutral pH, low ionic strength, and ambient temperature) bind α-thrombin, which elutes upon increasing the ionic strength (Fenton et al., 1981). Kaminski & McDonagh (1983) reported that thrombin binding to nonpolymerized fibrin agarose was reversible and saturable with $K_a = 4.9 \times 10^5$ M^{-1} depending on the thrombin/fibrin ratio. They also reported that the desorption of the enzyme from their resin was more pronounced by the divalent cation Ca^{2+} than monovalent cation Na^+ at corresponding ionic strengths. However, since $CaCl_2$ is a mild denaturant of human α-thrombin, our experiments employed an NaCl gradient to evaluate α-thrombin–fibrin binding (Landis et al., 1981). Since the active site of α-thrombin can be partitioned into a general apolar binding region (Berliner & Shen, 1977; Sonder & Fenton, 1984) and an anionic binding region (Conery & Berliner, 1983), we investigated whether these sites might participate in binding to nonpolymerized fibrin intermediates and presumptively to the fibrinogen recognition site. These studies strongly suggest that such fibrin interactions involve an anionic binding site.

Materials and Methods

Materials. Serotonin (creatinine) (lot 44C-1634), tryptamine hydrochloride (lot 93F-0146), creatinine sulfate (lot 26C-5004), and aspartame[1] (lot 122F-0884) were purchased from Sigma Chemical Co. (St. Louis, MO); DL-tryptophan was from Eastman Chemicals (Rochester, NY); bovine fibrinogen (lot 92, 75% clottable) was from Pentex; and Sepharose 4B was from Pharmacia.

Human α-thrombin was prepared and evaluated as described elsewhere (Fenton et al., 1977). The preparation used was >98% α-thrombin, titrated with NPGB to 95–98% active sites, and had a specific clotting activity of ~3000 NIH units/mg of protein. It was stored in 0.75 M NaCl at −72 °C and thawed immediately before use. Protein concentrations were estimated spectrophotometrically at 280 nm by assuming $E_{280} = 1.83$ mL mg^{-1} cm^{-1} for human α-thrombin in 0.1 M NaOH (Fenton et al., 1977). Clotting activities were expressed in NIH units by referencing with standardized human thrombin (lot J, kindly supplied by Dr. David L. Aronson, National Center for Drugs and Biologics, Bethesda, MD), as described elsewhere (Conery & Berliner, 1983). All other chemicals were reagent-grade, and all solutions were made with double-distilled water.

Preparation of Nonpolymerized Fibrin–Sepharose. Fibrinogen was immobilized on CNBr-activated agarose by a modification of the method of Heene & Matthias (1973). Briefly, ~2 g of CNBr was dissolved in 3 mL of cold acetonitrile and then added dropwise to 10 g (wet weight) of Sepharose 4B washed with 1000 mL of water and then washed with 50 mL of 2 M ice-cold phosphate buffer (pH 12.0). The reaction was allowed to proceed for 20 min at ice-bath temperature. The activated resin was washed with 1200 mL of cold 0.1 M sodium bicarbonate buffer, which was followed by 400 mL of 0.1 M sodium phosphate buffer (pH 7.8), and resuspended in 10 mL of sodium phosphate buffer (pH 7.8) in a new 125-mL polyethylene flask or bottle. Then, 0.3 g of fibrinogen was added in 30 mL of sodium phosphate buffer (pH 7.8); the bottle was flushed with N_2, sealed, and gently stirred overnight at 4 °C. In order to block unreacted remaining groups, the coupled fibrinogen resin was packed in a disposable plastic syringe and washed with the following buffers: 300 mL of 0.1 M sodium phosphate buffer (pH 7.8), 400 mL of 1 M NaCl in 0.1 M Tris-HCl (pH 7.8), 900 mL of 0.1 M glycine in 0.1 M $NaHCO_3$ (overnight), and finally 300 mL of 0.15 M NaCl in 0.1 M Tris-HCl (pH 7.6). Finally, 2 g of the washed fibrinogen resin in 15 mL of 0.15 M NaCl (0.1 M Tris, pH 7.6) was converted to the fibrin resin with α-thrombin at a final concentration of 0.1 unit/mL. The reaction was allowed to proceed for 3 h with gentle stirring at 20 °C (Heene & Matthias, 1973). (An apparent viscosity change of the solution was observed.) Finally, the nonpolymerized fibrin–Sepharose was then washed with the following 50 mM Tris-HCl (pH 7.6) buffer solutions: 200 mL of 3 M NaCl, 200 mL of 0.15 M NaCl, and 200 mL of 0.05 M NaCl.

Fibrin–Sepharose Column Chromatography. The above nonpolymerized fibrin resin was estimated to contain 47 mg of fibrin units/2 g (wet weight) of Sepharose 4B. The resin was packed in a plastic polypropylene Econo column (Bio-Rad, 0.8 × 4 cm, bed volume 2 mL) and equilibrated with 50 mM Tris–50 mM NaCl (pH 7.6). A 30–100-μL α-thrombin stock solution was then applied and the column washed with the preceding buffer. Elution was then carried out with a linear NaCl gradient (0.05–0.3 M) at 22 °C with a constant flow rate of 20–25 mL/h. Each fraction was collected in a plastic tube and assayed for clotting activity. Experiments were performed in the presence or absence of various ligands in the elution gradient. The conductivity of each fraction was measured and then converted to corresponding NaCl concentrations of the same conductivity. Apparent dissociation constants, K_d(app), were fit by nonlinear regression analysis.

Results and Discussion

The present experiments were carried out with an α-thrombin to fibrin molar ratio of 1:720, which required 170 ± 5 mM ($n = 6$) NaCl for midpeak elution of the enzyme from the resin (Figure 1a). In screening experiments, the concentration of NaCl required was found to follow a hyperbolic relationship when plotted against the fibrin to thrombin ratio (data not shown), which was similar to that reported for $CaCl_2$ elution (Kaminski & McDonagh, 1983). When 10 mM serotonin, an effective inhibitor of thrombin clotting or esterolytic/amidolytic activity (Conery & Berliner, 1983), was included in the gradient buffer, the enzyme eluted within ±1 standard deviation of the mean control (Figure 1b). The other elution profiles in Figure 1 depict the elution behavior of α-thrombin with selected thrombin inhibitors included in the gradient buffer. In contrast to serotonin, increasing concentrations of ATP or PP_i caused the enzyme to elute at much lower NaCl concentrations (Figure 1c,d), respectively; at 4 mM ATP, elution occurred before the salt gradient was initiated. A complete tabulation of the ligands examined in this study are listed in Table I.

In a more detailed study of the more potent ligands in Table I, the NaCl concentration for eluting α-thrombin was plotted

[1] Abbreviations: aspartame (Asp-Phe-OMe), L-aspartyl-L-phenylalanine methyl ester; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; P_i, phosphate; PP_i, pyrophosphate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

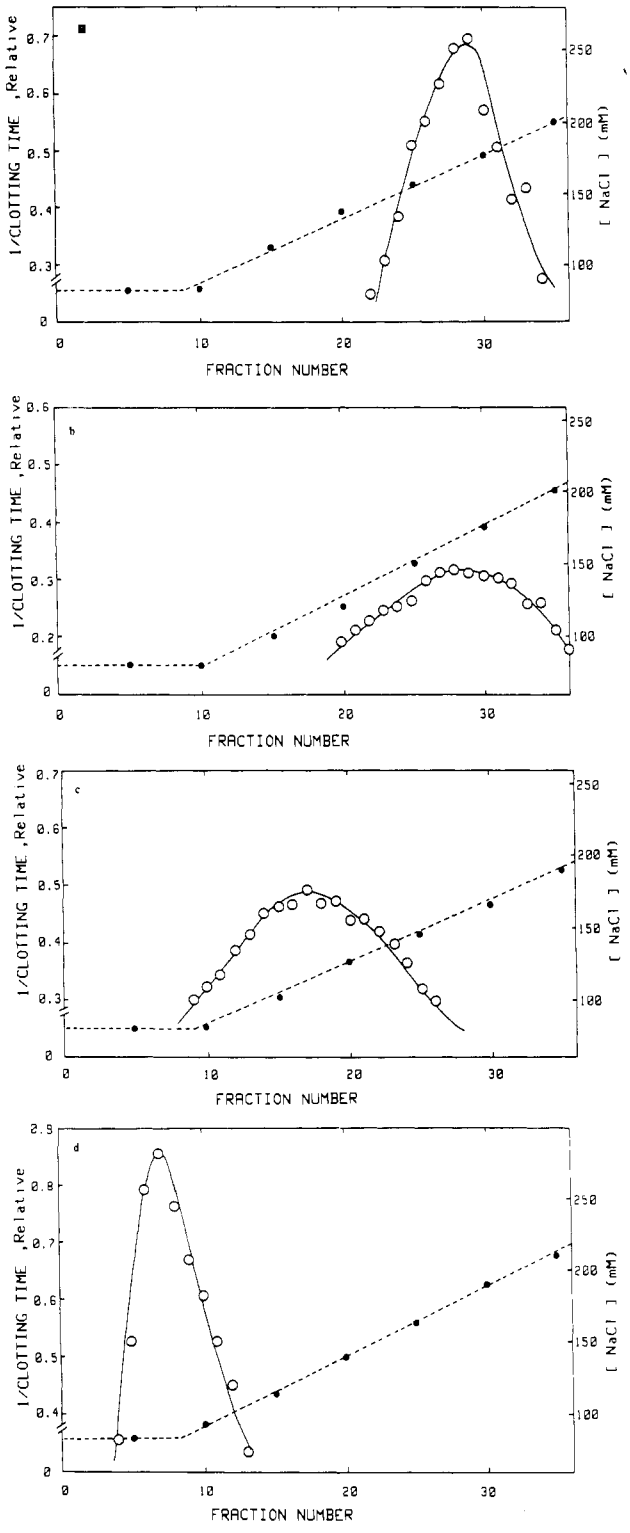

FIGURE 1: Elution profiles of human α-thrombin from nonpolymerized fibrin–Sepharose 4B in the presence of several ligands. The column was equilibrated at 50 mM Tris-HCl–50 mM NaCl, pH 7.6, followed by an NaCl gradient in the same buffer. Experiments were performed at a constant fibrin monomer/thrombin mole ratio of 720 at 22 °C. The gradient elution buffer also contained (a) control, (b) 10 mM serotonin, (c) 2 mM ATP, and (d) 10 mm PP_i.

vs. varying ATP (○), PP_i (△), or P_i (□) concentrations. An apparent dissociation constant, K_d(app), was estimated from each curve: 1.8 mM for ATP, 3.5 mM for PP_i, and 20 mM for P_i. Further examination of Table I indicates that the nucleotide ADP was clearly similar to ATP while citrate and oxalate (which are isoelectronic to PP_i) affected the desorption of α-thrombin from the fibrin resin in similar manners (Table I). Such effects were not due to the chelation of Ca^{2+} ion from the fibrin, since inclusion of 1 mM EGTA (which is adequate to remove all Ca^{2+} from the fibrin) did not effect the desorption of α-thrombin from the fibrin column. The K_d(app) values derived from these fibrin column experiments (Figure 2) were in remarkable agreement with the apparent equilibrium constants derived from inhibition or activation of estero/amidolytic or clotting activity and proflavin displacement measurements (Conery & Berliner, 1983; J. E. Scheffler and L. J. Berliner, unpublished results). That is, the similarity between the fibrin resin binding data and activity measurements strongly suggests that the ligand/inhibitor binding effects were at identical sites in each case.

Table I: Summary of Nucleotide Analogues, Serotonin Analogues, and Other Ligands on the Elution Profile of α-Thrombin from Nonpolymerized Fibrin–Sepharose by Increasing NaCl in 50 mM Tris-HCl, pH 7.6, 22 °C

compd (mM)	eluted by NaCl (mM)	compd (mM)	eluted by NaCl (mM)
control	170 ± 5	tryptophan analogues	
nucleotide analogues		serotonin (HCl)	
ATP		10	165
0.5	170	tryptamine (HCl)	
1	155	10	150
2	115	20	175
4	80	DL-tryptophan	
10	80	10	160
ADP		creatinine sulfate	
10	80	5	150
PP_i		10	150
2	145	20	140
4	125	caffeine	
6	110	10	150
10	95	35	140
15	95	miscellaneous substances	
phosphate		EGTA	
10	160	1	175
20	145	benzamidine	
30	120	1.5	160
50	125	$NaHCO_3$	
sodium citrate		10	160
10	125	aspartame	
20	110	10	160
sodium oxalate			
10	155		
30	135		
50	125		

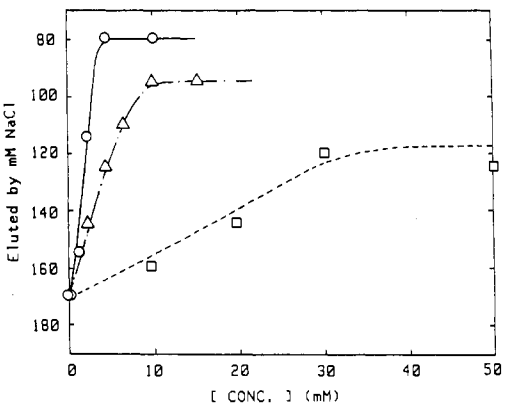

FIGURE 2: Ionic strength (measured in units of NaCl concentration) for the midpeak elution of α-thrombin in the presence of increasing concentrations of ATP (○), PP_i (△), and P_i (□). Apparent dissociation constants, K_d(app), were calculated as 1.8 mM for ATP, 3.5 mM for PP_i, and 20 mM for P_i. All other conditions were identical with those in Figure 1.

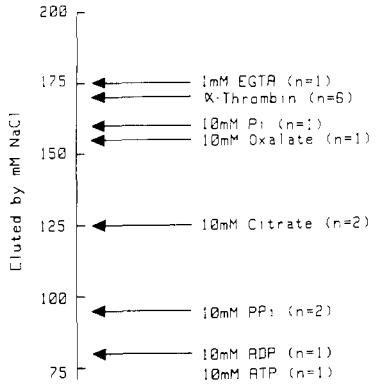

FIGURE 3: Effect of ATP analogues and related anionic substances on the interaction between α-thrombin and nonpolymerized fibrin. The NaCl concentration (±5 mM) for elution is indicated by an arrow in each case. The value in parentheses indicates the number of trials.

Catalytically inactivated forms of α-thrombin that are conjugated at the active serine (with e.g., diisopropyl phosphorofluoridate, phenylmethanesulfonyl fluoride, or methanesulfonyl fluoride) bind to nonpolymerized fibrin resin but elute at salt concentrations similar to that of the control (native enzyme). Furthermore, the essentially nonclotting γ-thrombin form of the human enzyme exhibits markedly reduced binding, while the hirudin complex of this and the α form does not bind to the resin at all (Fenton et al., 1981). Thus, the fibrin binding site appears to not involve the catalytic site (i.e., the immediate active site) but instead a region related to other requirements for clotting activity (fibrinogen or fibrin binding). As noted in the present studies (Table I), a concentration of 1.5 mM benzamidine had essentially no effect on the elution of α-thrombin, while this compound has an inhibition constant of 0.22 mM against both fibrinogen clotting esterolytic/amidolytic activities with synthetic substrates (Markwardt et al., 1968).

The tryptophan analogues serotonin and tryptamine at 5–20 mM concentrations, are also inhibitors of clotting, as well as effectors of thrombin-catalyzed hydrolysis of various synthetic substrates (Conery & Berliner, 1983). These apolar compounds bind at (or near) the proflavin apolar binding site (Berliner & Shen, 1977), which is also conserved in nonclotting γ-thrombin (Conery & Berliner, 1983) and has been proposed to reside in the fibrinopeptide groove adjacent to the catalytic site (Sonder & Fenton, 1984). The present findings that 10 and 20 mM concentrations of various tryptophan analogues did not effect α-thrombin binding to the fibrin resin (Table I) imply that this site is not involved in binding to nonpolymerized fibrin. Likewise, various concentrations of other tryptophan analogues and miscellaneous substances had no significant effects.

In contrast, the marked inhibitory effects of the negatively charged nucleotides, ADP and ATP, as well as the effects of other anionic substances, particularly PP_i, suggest that the fibrin binding region involves an anionic site. While all of these compounds quite effectively inhibit clotting activity, PP_i (and P_i) does not appreciably inhibit synthetic substrate activities (Berliner, 1984; J. E. Scheffler and L. J. Berliner, unpublished results). Thus, this anionic binding site appears to be independent of the catalytic center as also verified by the failure of active site inhibitors to effect α-thrombin desorption from the fibrin resin. Figure 3 pictorially depicts the results with anionic ligands.

These results are based on the assumption that the fibrin–Sepharose resin is indeed predominantly nonpolymerized fibrin. Other investigators have shown that the coupling of fibrinogen to CNBr-activated agarose occurs principally through the Aα chain (Matthias et al., 1974) and the resultant fibrinogen–agarose is converted to monomeric fibrin conjugate, which binds fibrinogen (Heene & Matthias, 1973; Kaminski & McDonagh, 1983). While it is not possible to describe in detail how all of the fibrin monomers are attached to the Sepharose matrix or whether all possess identically accessible binding sites for thrombin, this limitation does not detract from the results presented here. Kaminski & McDonagh (1983) estimated that approximately 10% of the bound nonpolymerized fibrin have functional binding sites for fibrinogen. We employed a very low thrombin to fibrin molar ratio (1:720) in these studies. Furthermore, several other lines of evidence point to the fact that thrombin–fibrin interactions occur principally with monomers. Radioiodinated (^{125}I) α-thrombins were found to incorporate into fibrin but only during an *intermediate* phase of the active polymerization process, i.e., binding to nonpolymerized fibrin (Wilner et al., 1981). The enzyme does not photoaffinity label fibrin in clots (Carney et al., 1979), and the free active enzyme is readily *released* upon fibrinolysis. Electron micrographs of colloidal gold-labeled thrombin complexes with fibrin gels revealed thrombin–fibrin association *only* at branch points—most likely to contain accessible end structures or free monomers (Liu et al., 1985).

The physiological implications of these results may relate significantly to fibrin(ogen) recognition and binding in clot formation. The observations that radiolabeled photoaffinity-labeled α-thrombin does not cross-link with fibrin when photoactivated after clotting (Carney et al., 1979) and that active-site conjugated spin-labeled α-thrombin does not display an immobilized ESR spectrum in a fibrin clot like the hirudin α-thrombin complex (Fenton et al., 1979) suggest that the enzyme is not bound to clotted fibrin. Thus, it follows that α-thrombin must interact with the nonpolymerized fibrin units (monomers) in order to become actively incorporated into the fibrin clot (Wilner et al., 1981). Furthermore, while *physical* measurements of thrombin–fibrinogen interactions are extremely difficult, if not impossible, to measure without the complications of fibrin monomer and clot formation, the correlation between clotting inhibition and thrombin–fibrin monomer binding (Figure 2), as well as preliminary studies of inactivated thrombin–fibrinogen–Sepharose binding (Fenton et al., 1981), suggests that similarities exist between α-thrombin–fibrin and –fibrinogen interactions. Lastly, this anionic binding site may be involved in hirudin binding as well as fibrinogen recognition.

Registry No. 5′-ATP, 56-65-5; ADP, 58-64-0; pyrophosphate, 14000-31-8; phosphate, 14265-44-2; citrate anion (3−), 126-44-3; oxalate anion (2−), 338-70-5; α-thrombin, 9002-04-4.

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Regulation of the Adenosinetriphosphatase Activity of Cross-Linked Actin–Myosin Subfragment 1 by Troponin–Tropomyosin

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Received April 3, 1985

ABSTRACT: Chalovich and Eisenberg [Chalovich, J. M., & Eisenberg, E. (1982) *J. Biol. Chem. 257*, 2432–2437] have suggested that at low ionic strength, troponin–tropomyosin regulates the actomyosin ATPase activity by inhibiting a kinetic step in the actomyosin ATPase cycle rather than by blocking the binding of myosin subfragment 1 (S-1) to actin. This leads to the prediction that troponin–tropomyosin should inhibit the ATPase activity of the complex of actin and S-1 (acto·S-1) even when S-1 is cross-linked to actin. We now find that the ATPase activity of cross-linked actin·S-1 prepared under milder conditions than those used by Mornet et al. [Mornet, D., Bertrand, R., Pantel, P., Audemard, E., & Kassab, R. (1981) *Nature (London) 292*, 301–306] is inhibited 90% by troponin–tropomyosin in the absence of Ca^{2+}. At μ = 18 mM, 25 °C, the ATPase activity of this cross-linked preparation is only about 2-fold greater than the maximal actin-activated ATPase activity of S-1 obtained with regulated actin in the absence of Ca^{2+}. At physiological ionic strength, the ATPase activity of this cross-linked actin·S-1 preparation is inhibited about 95% by troponin–tropomyosin. Since cross-linked S-1 behaves kinetically like S-1 in the presence of infinite actin concentration, it is very unlikely that inhibition of the ATPase activity of cross-linked actin·S-1 is due to blocking of the binding of S-1 to actin. Therefore, these results are in agreement with the suggestion that troponin–tropomyosin regulates primarily by inhibiting a kinetic step in the ATPase cycle.

Vertebrate skeletal muscle contraction is controlled by the Ca^{2+} concentration in muscle; at low levels of Ca^{2+} ($<10^{-6}$ M^{-1}), the muscle is relaxed, and the associated actin-activated ATPase activity of the myosin cross-bridges is inhibited, while increasing the Ca^{2+} concentration reverses this process. This regulation by Ca^{2+} is mediated by the regulatory complex troponin–tropomyosin (Perry, 1979; Weber & Murrary, 1973). Tropomyosin lies along the actin groove of the thin filament, with each tropomyosin molecule binding to seven actin monomers (Ebashi, 1980). Bound to one end of tropomyosin is a troponin molecule, composed of three subunits, one of which binds Ca^{2+}. X-ray diffraction studies have shown that the binding of Ca^{2+} causes a shift in the position of the tropomyosin relative to the actin groove; in the presence of Ca^{2+}, tropomyosin lies in the actin groove, while in the absence of Ca^{2+}, tropomyosin moves away from the groove to a position in which it may be able to interfere with the binding of the myosin cross-bridge on actin (Haselgrove, 1972; Huxley, 1972; Parry & Squire, 1973). These structural data formed the basis for the steric blocking model which suggests that relaxation is due to tropomyosin physically blocking the binding of the myosin cross-bridge to actin. In a variant of this model, it has also been suggested that troponin–tropomyosin may indirectly block the binding of the cross-bridge to actin by inducing a conformational change in the actin itself (Weber & Murray, 1973).

Recently, the steric blocking model was tested by measuring the binding of myosin subfragment 1 (S-1)[1] to regulated actin

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[1] Abbreviations: S-1, myosin subfragment 1; acto·S-1, complex of actin and S-1; regulated acto·S-1, complex of troponin–tropomyosin and acto·S-1; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; IAN-BD, 4-[*N*-[(iodoacetoxy)ethyl]methylamino]-7-nitro-2,1,3-benzoxadiazole; HMM, heavy meromyosin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate; DTT, dithiothreitol.