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Fabrice Cornille, Loïc M Martin, Christine Lenoir, Didier Cussac, Bernard P. Roques, et al.. Cooperative Exosite-dependent Cleavage of Synaptobrevin by Tetanus Toxin Light Chain. *Journal of Biological Chemistry*, 1997, 272 (6), pp.3459-3464. 10.1074/jbc.272.6.3459 . hal-02528515

HAL Id: hal-02528515

<https://hal.science/hal-02528515v1>

Submitted on 1 Jun 2021

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Cooperative Exosite-dependent Cleavage of Synaptobrevin by Tetanus Toxin Light Chain*

(Received for publication, April 30, 1996, and in revised form, November 21, 1996)

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The light chain (L chain) of tetanus neurotoxin (TeNT) has been shown to have been endowed with zinc endopeptidase activity, selectively directed toward the Gln⁷⁶-Phe⁷⁷ bond of synaptobrevin, a vesicle-associated membrane protein (VAMP) critically involved in neuroexocytosis. In previous reports, truncations at the NH₂ and COOH terminus of synaptobrevin have shown that the sequence 39–88 of synaptobrevin is the minimum substrate of TeNT, suggesting either the requirement of a well defined three-dimensional structure of synaptobrevin or a role in the mechanism of substrate hydrolysis for residues distal from the cleavage site. In this study, the addition of NH₂- and COOH-terminal peptides of synaptobrevin, S 27–55 (S₁) and S 82–93 (S₂), to the synaptobrevin fragment S 56–81 allowed the cleavage of this latter peptide by TeNT to occur. This appears to result from an activation process mediated by the simultaneous binding of S₁ and S₂ with complementary sites present on TeNT as shown by surface plasmon resonance experiments and the determination of kinetic constants. All these results favor an exosite-controlled hydrolysis of synaptobrevin by TeNT, probably involving a conformational change of the toxin. This could account for the high degree of substrate specificity of TeNT and, probably, botulinum neurotoxins.

have been found (9). These L chains, but not their apo-forms, cleave specifically three proteins of the neuroexocytosis apparatus: synaptobrevin, which is hydrolyzed by TeNT, BoNT/B, /D, /F, and /G, SNAP 25, which is cleaved by BoNT/A, /C₁, and /E, and syntaxin by BoNT/C₁ (10–12). Moreover, as suspected from previous studies on zinc metallopeptidases (13), site-directed mutagenesis of the recombinant light chains of TeNT and BoNT/A showed that the two histidines of the motif His-Glu-Xaa-Xaa-His are zinc ligands and the glutamate plays a critical role in the catalytic process (14–17).

Among the group of zinc metallopeptidases, a particularity of clostridial neurotoxins is a very high substrate selectivity, contrasting with the wide specificity of zinc proteases belonging to the thermolysin and metzincin families (13). Indeed, except for BoNT/C₁ (11, 12), the clostridial neurotoxins appear to cleave a single protein substrate at a single peptide bond (10). The target of five clostridial neurotoxins, synaptobrevin, also known as VAMP (for vesicle-associated membrane protein), is a protein highly conserved in eucaryotes (18–20), which play a central role in neuroexocytosis (21–27). Human synaptobrevin isoform II contains 116 residues with a short COOH-terminal tail anchoring the molecule into the membrane of small synaptic vesicles. Its cytosolic region is composed of a NH₂-terminal proline-rich domain of 24 residues followed by a hydrophilic domain of 72 amino acids (Fig. 1). TeNT cleaves synaptobrevin selectively at one single peptide bond Gln⁷⁶-Phe⁷⁷ (28). Successive truncations of synaptobrevin at its NH₂ and COOH terminus have shown that the removal of both the proline-rich NH₂-terminal 1–25 domain and the 93–116 transmembrane anchor does not reduce the rate of hydrolysis by TeNT (29, 30) (Fig. 1). Conversely, truncations of the 27–49 acidic domain (30–32) as well as truncations of the 82–93 basic domain (32) results in a dramatic decrease (100 times) in the rate of cleavage (Fig. 1), the minimum substrate of TeNT being the 50-residue peptide S 39–88 (32). Furthermore, several copies of a common acidic motif are present in the sequence of the three targets of clostridial neurotoxins (33). Antibodies raised against this motif cross-react with synaptobrevin, SNAP-25, and syntaxin and inhibit the cleavage of synaptobrevin by either TeNT or BoNT/B or /G (34). Two copies of this acidic motif are present in the sequence of synaptobrevin and correspond to the 38–47 and 62–71 regions designated V1 and V2, respectively. Substitutions of the acidic residues of V1 by serines in synaptobrevin abolish almost completely its cleavage by TeNT (34, 35). Altogether these findings suggested that domains of synaptobrevin far from the cleavage site could play an important role in the hydrolysis of this peptide either by inducing a cleavable conformation of synaptobrevin at the Gln⁷⁶-Phe⁷⁷ bond or by promoting a proteolytic conformation of the TeNT L chain. With the aim of answering these questions,

Tetanus toxin (TeNT)¹ and the seven serotypes of botulinum neurotoxins (BoNT/A, /B, /C, /D, /E, /F, and /G), which are produced by several anaerobic bacilli of the genus *Clostridium*, cause the paralytic syndromes of tetanus and botulism by blocking neurotransmitter release at central and peripheral synapses, respectively (1, 2). They are formed by two disulfide-linked polypeptides, the heavy (H) chain (100 kDa) being responsible for specific binding to neurons and cell penetration, and the light (L) chain (50 kDa) for neuroexocytosis blockade, the reduction of the disulfide bond being required for this process (1, 2). The L chain of these neurotoxins contains the typical His-Glu-Xaa-Xaa-His sequence of zinc endopeptidases (3–5). Accordingly, each toxin has been shown to contain one atom of zinc (6–8), except BoNT/C for which two zinc atoms

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¹ The abbreviations used are: TeNT, tetanus toxin; S, synaptobrevin; BoNT, botulinum neurotoxin; L chain, light chain; H chain, heavy chain; RP, reverse phase; HPLC, high performance liquid chromatography; RU, resonance units; Pya, pyrenylalanine.

and the reaction mixture was then loaded on a Sep-Pak C₁₈ cartridge (Waters) in order to separate the fluorescent cleavage product [Pya⁸⁸]S 77–88 from the substrate. This metabolite was eluted with 3 ml of 65% MeOH, 0.1% trifluoroacetic acid in H₂O, and the fluorescence of the eluate was read at 377 nm after excitation of the fluorophore at 343 nm using a Perkin-Elmer LS-50B fluorometer. The amount of released cleavage product was calculated from the fluorescence of known concentrations of the synthetic cleavage product [Pya⁸⁸]S 77–88. The same assay was used for dose-dependent experiments with S₁ and S₂ peptides and to determine the kinetic constants for enzymatic cleavage in the presence of 100 μM S₁ and S₂ peptides. In some cases, enzymatic reactions were stopped by addition of 1 μl of trifluoroacetic acid, and the mixture was analyzed by RP-HPLC on a Capcell C₈ (300 Å, 5 μm, 250 × 4.6 mm) column by using a 25–45% B gradient (A, trifluoroacetic acid 0.05% in H₂O; B, CH₃CN 90%, trifluoroacetic acid 0.038% in H₂O) with an ultraviolet detection at 343 nm, in order to verify that the fluorescence increase was exclusively related to the formation of the cleavage product [Pya⁸⁸]S 77–88.

RESULTS

Promoting Effects of Peptides S₁ or S₂ on the Cleavage of Fragments S 50–93, S 32–81, and S 56–81 by TeNT L Chain—To determine whether S₁ or S₂ could promote the cleavage of synaptobrevin fragments S 50–93, S 32–81, and S 56–81, which are poorly or not at all cleaved by TeNT L chain, the latter peptides were incubated in the presence of 1 mM S₁ or S₂ before addition of TeNT L chain. After 20 min at 37 °C, the reaction mixtures were analyzed by RP-HPLC in order to quantify the level of cleavage products generated. No effect on the cleavage rate of the optimal substrate S 32–93 was observed after addition of the same concentrations of S₁ (Fig. 2, C and D) or S₂ (Fig. 3A). In contrast, the cleavage rate of S 50–93 in the presence of 1 mM S₁ increased 34-fold (52 pmol·min⁻¹·μg⁻¹) reaching a level comparable to that of S 32–93 (102 pmol·min⁻¹·μg⁻¹) (Fig. 2, A and B) but was not enhanced by the addition of 1 mM S₂ (Fig. 3B). An even greater potentiating effect was observed following addition of 1 mM S₂ to S 32–81 (Fig. 3C). This effect is difficult to quantify because, in the absence of S₂, the cleavage of S 32–81 was undetectable; however, taking 0.1 pmol·min⁻¹·μg⁻¹ as the limit of cleavage product detection, it can be assumed to be greater than 150-fold. The addition of 1 mM S₁ to this peptide did not improve its cleavage (Fig. 3C). In view of these results the influence of the addition of 1 mM S₁ and/or 1 mM S₂ was tested on the synaptobrevin fragment S 56–81, lacking both S₁ (S 27–55) and S₂ (S 82–93) domains. In this case, as expected, the simultaneous presence of the two peptides S₁ and S₂ allowed a significant TeNT enzyme activity (12 pmol·min⁻¹·μg⁻¹) (Fig. 3D) to be observed. Interestingly, no effects could be obtained under the same conditions with a peptidic sequence shorter than S₁, S 37–47 (data not shown), which corresponds to the acidic motif conserved in SNAP-25 and syntaxin (33).

The Presence of S₂ Is Required to Observe TeNT L Chain Binding to S₁—A convenient method to observe interactions between small soluble peptides and proteins is to use the surface plasmon resonance technique (41). When a 6 μM solution of TeNT L chain was injected into a flow cell containing immobilized S₁, no binding was observed (Fig. 4). Contrastingly, injection of a mixture of 6 μM TeNT L chain with 50 μM S₂ showed a specific binding of about 4000 RU (Fig. 4). In a control experiment, injection of 50 μM peptide S₂ alone was shown to produce only a small response of 330 RU (Fig. 4). The S₂-promoted interaction between TeNT and S₁ was dose-dependently reversed by preincubating the TeNT-S₂ mixture with a large excess of S₁. With 1 mM S₁, no detectable binding of TeNT was observed (Fig. 4, inset).

Determination of the Influence of S₁ and S₂ on the Kinetic Constants of the Enzymatic Activity of TeNT L Chain—The minimum fluorescent substrate [Pya⁸⁸]S 39–88, which con-

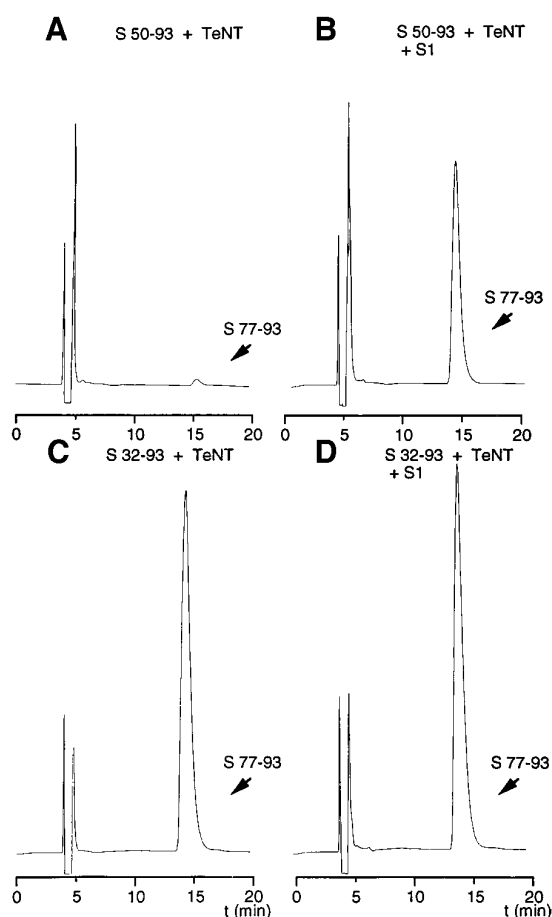


Fig. 2. HPLC chromatograms showing the influence of the addition of S₁ on the cleavage of S 50–93 and S 32–93 by TeNT L chain. 100 μM S 50–93 or S 32–93 were incubated in presence or in absence of 1 mM S₁ in 100 μl of 20 mM Hepes, pH 7.4, with 1 μg of TeNT L chain (200 nM) for 20 min at 37 °C. The reaction was stopped by addition of 50 μl of 0.2 N HCl. The released metabolite S 77–93 was quantified by RP-HPLC on a Capcell C₈ column (300 Å, 7 μm, 250 × 4.6 mm) in isocratic conditions (24% B for 20 min; A, 0.05% trifluoroacetic acid in H₂O; B, 90% CH₃CN, 0.038% trifluoroacetic acid in H₂O; ultraviolet detection: λ = 214 nm).

tains the non-natural fluorescent amino acid pyrenylalanine (Pya) in position 88 (32) was chosen to study the influence of S₁ and S₂ on the kinetic constants of the enzymatic reaction. Increasing concentrations of peptides S₁ and S₂ induced a dose-dependent increase in [Pya⁸⁸]S 39–88 cleavage, with maximal substrate degradation obtained at 100 μM either S₁ or S₂ (170 and 600%, respectively) (Fig. 5), which was therefore the concentration used to measure the influence of the peptides on the kinetic constants of the enzymatic reaction (Table I). The addition of peptide S₂ produced a shift of the apparent Michaelis constant (K_m) value of the substrate from 576 to 51 μM, while the maximal velocity (V_{max}) was only slightly affected (166 instead of 111 pmol·min⁻¹·μg⁻¹). As expected from a partial overlap of the sequences of S₁ and the [Pya⁸⁸]S 39–88 substrate, the potentiating effect induced by the peptide S₁ was lower, the K_m shifting from 576 to 479 μM and the V_{max} shifting from 111 to 169 pmol·min⁻¹·μg⁻¹ (Table I). In all these studies, control experiments by RP-HPLC indicated that the enhanced fluorescence corresponded to an increase in the formation of the cleavage product [Pya⁸⁸]S 77–88 (data not shown). No other cleavage was observed.

DISCUSSION

The purpose of this study was to investigate the mechanism involved in the highly specific and selective cleavage of synap-

FIG. 3. Influence of the addition of S_1 or/and S_2 on the enzymatic cleavage of different synaptobrevin peptides by TeNT L chain. 10^{-4} M peptide substrate (10^{-3} M in the case of S 56–81) were incubated in presence or in absence of 1 mM S_1 or/and 1 mM S_2 in a total volume of 100 μ l of 20 mM Hepes pH 7.4 with 1 μ g of TeNT L chain at 37 °C. The reaction was stopped by addition of 50 μ l of 0.2 N HCl. The released cleavage products S 77–93 or S 77–81 were quantified by RP-HPLC as described under "Materials and Methods" and in Fig. 2. Cleavage rate is expressed as \pm S.E. of three independent experiments performed in duplicate.

Hydrolysis of Synaptobrevin fragments by TeNT				Cleavage rate (pmoles.min ⁻¹ . μ g ⁻¹)
		TeNT Cleavage		
		32	93	
		LQQTQAQVDEVVDIMRVNDKVLERDQKSELDDRADALQAGASQFETSAAKLRKRYWVKNL		
A	27	S1	55	102 \pm 5
		NLTNSRRRLQQTQAQVDEVVDIMRVNDKVLE + S 32-93		105 \pm 5
		S 32-93 + 82 AKLRKRYWVKNL 93		103 \pm 5
		50	93	
		VDKVLERDQKSELDDRADALQAGASQFETSAAKLRKRYWVKNL		
B	27	S1	55	1.5 \pm 0.5
		NLTNSRRRLQQTQAQVDEVVDIMRVNDKVLE + S 50-93		52 \pm 2
		S 50-93 + 82 AKLRKRYWVKNL 93		1.5 \pm 0.5
		32	81	
		LQQTQAQVDEVVDIMRVNDKVLERDQKSELDDRADALQAGASQFETS		
C	27	S1	55	< 0.1
		NLTNSRRRLQQTQAQVDEVVDIMRVNDKVLE + S 32-81		< 0.1
		S 32-81 + 82 AKLRKRYWVKNL 93		15 \pm 0.5
		56	81	
		RDQKSELDDRADALQAGASQFETS		
D	27	S1	55	< 0.1
		NLTNSRRRLQQTQAQVDEVVDIMRVNDKVLE + S 56-81		< 0.1
		S 56-81 + 82 AKLRKRYWVKNL 93		< 0.1
		S 56-81 + 82 AKLRKRYWVKNL 93		12 \pm 0.5

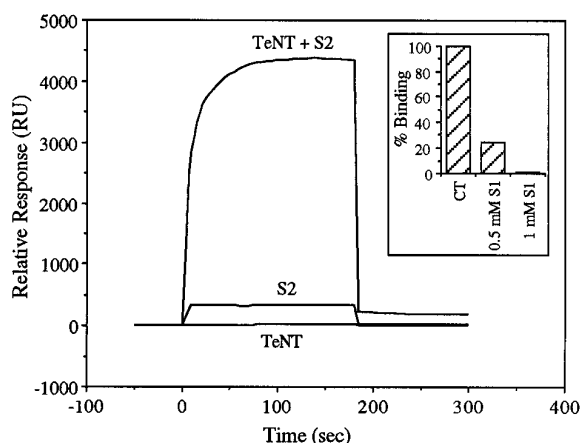


FIG. 4. Binding of TeNT-L chain to S_1 in presence of S_2 observed by surface plasmon resonance (BIAcore). RU are plotted as a function of time (sensorgrams) for representative experiments. 30 μ l of 6 μ M TeNT L chain in 20 mM Hepes, pH 7.4 (bottom trace), or 30 μ l of 50 μ M S_2 in 20 mM Hepes, pH 7.4 (middle trace), or 30 μ l of 6 μ M TeNT L chain + 50 μ M S_2 in 20 mM Hepes, pH 7.4 (top trace), were passed into a flow cell coated with S_1 , at a flow rate of 10 μ l/min. The 0 abscissa represents the beginning of injection. The inset represents the abolition of TeNT binding observed by coinjection of TeNT + S_2 with increasing concentrations of S_1 . The relative response expressed in percentage of binding was calculated at equilibrium ($t = 150$ s after injection)

tobrevin at the Gln⁷⁶-Phe⁷⁷ bond (28). Previously reported data have shown that the acidic NH₂-terminal sequence 27–55 and the basic COOH-terminal sequence 82–93 of synaptobrevin are essential for an efficient cleavage of this peptide (29–32). Different possibilities could account for these data. (i) The hydrolysis of synaptobrevin could require a well defined tertiary structure of the peptide substrate induced by residues far from the cleavage site. (ii) Additional interactions with TeNT could be necessary to generate the Michaelis complex. (iii) Interactions of well defined sequences of the substrate with specific exosites present on tetanus toxin could be necessary to promote synaptobrevin cleavage, as it is the case for allosteric enzymes.

In order to answer these questions, synthetic peptides S_1 and S_2 corresponding, respectively, to the acidic and basic motif of synaptobrevin were tested for their ability to enhance the cleavage rate of the complementary synaptobrevin sequences

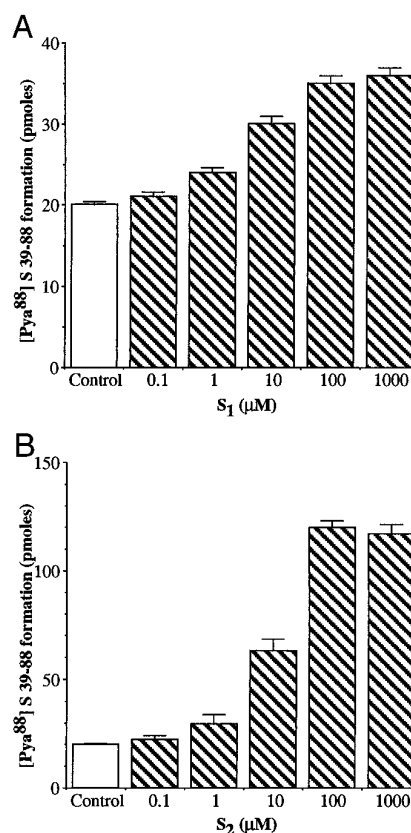


FIG. 5. Dose-dependent activation of TeNT L chain endopeptidase activity by synaptobrevin fragments S_1 and S_2 . 20 μ M [88 Pya]S 39–88 were incubated in presence of different concentrations of S_1 (A) and S_2 (B) in 100 μ l of 20 mM Hepes, pH 7.4, with 250 ng of TeNT L chain for 20 min at 37 °C in darkness. The released cleavage product [88 Pya]S 77–88 was separated from the substrate and quantified by fluorescence spectrometry. Control represents the absence of modulatory peptide.

by TeNT. Interestingly, the cleavage of the synaptobrevin peptide S 50–93, which lacks almost completely the S_1 sequence and which is very poorly cleaved by TeNT, was greatly enhanced (34-fold) by the addition of 1 mM S_1 . Similarly, the cleavage of the synaptobrevin peptide S 32–81, in which the full sequence of S_2 is lacking and which is not cleaved by TeNT, was triggered (150-fold) by the addition of 1 mM S_2 . The deci-

TABLE I
Kinetic constants determination of [Pya⁸⁸]S 39-88 cleavage by TeNT L Chain: influence of either S₁ or S₂

100 μM of each peptides S₁ or S₂ were used in these experiments. These values represent the standard error of the mean of three independent experiments performed in duplicate (*p* < 0.01 Dunnett *t* test).

Peptide	<i>K_m</i>	<i>V_{max}</i>
[Pya ⁸⁸]S 39-88	576 ± 11	111 ± 10
[Pya ⁸⁸]S 39-88 + S ₁	479 ± 12	169 ± 11
[Pya ⁸⁸]S 39-88 + S ₂	51 ± 11	166 ± 10

sive demonstration of the role of the S₁ and S₂ motifs came from the cleavage of S 56-81, a synaptobrevin peptide lacking both S₁ and S₂ domains, which was induced by the simultaneous addition of 1 mM S₁ and 1 mM S₂.

In all these experiments the cleavage rate measured for the mixtures of peptides was 2-7 times lower than that measured with the optimum substrate S 32-93 (Fig. 3). This was not unexpected, since the affinity for TeNT of the isolated fragments and their ability to fit the complementary sites within the protein cannot be as high as when the binding motifs are included in the substrate sequence.

These results do not support the hypothesis of a role for the NH₂- and COOH-terminal peptides S₁ and S₂ in inducing a TeNT-cleavable conformation of synaptobrevin. Indeed, the formation of a unique tertiary structure, comparable to that of S 27-93, by mixing the three short peptides S 27-55 (S₁), S 56-81, and S 82-93 (S₂) is highly improbable. This is supported by the lack of a well defined tertiary structure for synaptobrevin in solution as previously observed by ¹H NMR (29). A mechanism which could account for the present results is the promotion of an active conformation of TeNT induced by the binding of the S₁ and S₂ domains of synaptobrevin to exosites present on the toxin surface, as in the case of allosteric enzymes (36-38). Surface plasmon resonance analysis clearly showed the binding of the S₁ domain of synaptobrevin to TeNT. This interaction occurs only in the presence of the S₂ fragment and is not due to a direct interaction between S₁ and S₂ as shown by ¹H NMR analysis (data not shown). This finding strongly suggests a cooperative interaction of both exosites. Conversely, when S₂ was immobilized on the sensor chip, the ionic interaction of the highly basic S₂ fragment with the multiple negative charges of the dextran matrix probably hampered the correct orientation of the S₂ side chains, accounting for the absence of binding observed when either TeNT or a TeNT-S₁ mixture was injected through a flow cell coated with S₂ (data not shown). However, this interaction has been clearly demonstrated by the 10-fold decrease in the *K_m* value of the fluorescent substrate following addition of the lacking S₂ fragment (Table I).

Altogether these findings suggest that peptides S₁ and S₂ favor the cleavage of synaptobrevin fragments by interaction with corresponding exosites present at the toxin surface and are in good agreement with an allosteric model of enzyme functioning (36-38). According to this hypothesis, the predominant conformational state of TeNT L chain in solution could correspond to nonproteolytic form(s) in equilibrium with a very low percentage of the proteolytic form. The higher affinity of the S₁ and S₂ domains for the latter form would increase its population, their interaction with the corresponding exosites on TeNT triggering the proteolytic cleavage of the vesicle-associated membrane protein (Fig. 6). The increased apparent affinity of the substrate [Pya⁸⁸]S 39-88, reflected by a reduction in *K_m* values in the presence of S₁ and especially S₂ while the maximal velocity is not significantly affected, also favors this assumption. Nevertheless, further physicochemical exper-

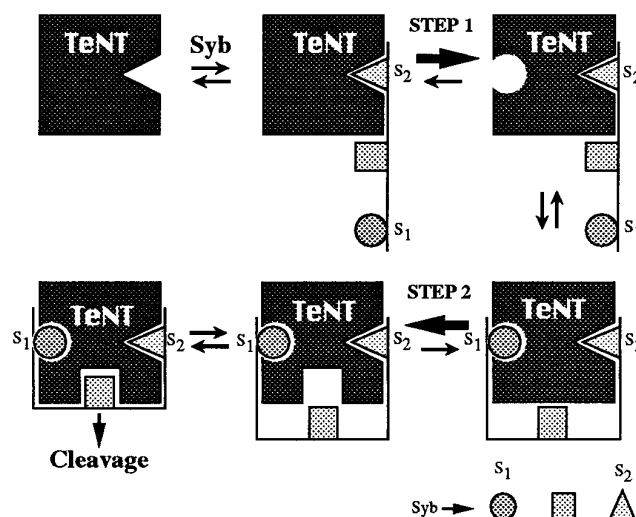


FIG. 6. Model of cooperative exosite-dependent cleavage of synaptobrevin by TeNT. Δ represents the S₂ sequence of synaptobrevin; \ominus the S₁ sequence, and \square the cleavage sequence. TeNT L chain might exist mainly under nonproteolytic conformation(s), which can bind the S₂ sequence of synaptobrevin. The S₂-TeNT complex undergoes a transformation (STEP 1), which allows TeNT to bind S₁. This induces the final conformational transition (STEP 2), and subsequent formation of the Michaelis complex.

iments will be necessary to physically observe the proposed change of TeNT conformation. As far as we know, a cooperative mechanism has never been described for zinc proteases and for proteases in general only for thrombin, a trypsin-like serine protease, which makes use of exosites for recognition of its substrate (42). TeNT L chain selectivity is dependent on this type of mechanism. Given the similar behavior of BoNT/B and /G toward synaptobrevin in terms of structure-activity relationships (34, 35), a similar mechanism could also be involved in the selectivity of other clostridial neurotoxins.

Acknowledgments—We thank Dr. J. R. Cartier (Pasteur-Mérieux) for kindly providing purified TeNT dichain and Dr. J. P. Changeux for critical reading of the manuscript.

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