Localization in diphtheria toxin fragment B of a region that induces pore formation in planar lipid bilayers at low pH

Michel Deleers, Nadine Beugnier+, Paul Falmagne+, Véronique Cabiaux* and Jean-Marie Ruysschaert*,°

Laboratoire de Médecine Expérimentale, Faculté de Médecine, 1000 Bruxelles, +Laboratoire de Chimie Biologique, Faculté des Sciences, Université de l'Etat à Mons, B-7000 Mons and **Laboratoire de Chimie Physique des Macromolécules aux Interfaces, Université Libre de Bruxelles, CP 206/2, Bd du Triomphe, 1050 Bruxelles, Belgium*

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Like diphtheria toxin and the N-terminal (M_r 23000) region of fragment B, CB1 (M_r13000), the cyanogen bromide peptide located in the middle region of fragment B is able to induce pore formation in lipid bilayer membrane at low pH. These two peptides (M_r 23000 and 13000) share a common segment (M_r 6300) containing the predicted amphipathic, α-helical, transverse lipid-associating domain (M_r 2750) of fragment B [J. Cell Biol. (1980) 87, 837–840]. Therefore, we postulated this domain to be responsible for the pore formation ability of diphtheria toxin [Proc. Natl. Acad. Sci. USA (1981) 78, 172–176]. A relationship between the pH dependency of pore formation and the presence of a cluster of prolines in the C-terminal region of CB1 is proposed.

Diphtheria toxin *Fragment B amphipathic region* *Lipid-associating domain* *Voltage-dependent conductance* *Proline isomerization*

1. INTRODUCTION

Diphtheria toxin (M_r 60000) kills sensitive eukaryotic cells in a series of steps involving binding of its C-terminal portion (fragment B; M_r 38000) to specific cell surface receptors at neutral pH, internalization into vesicles of still disputed nature, acidification of the vesicular medium followed by fragment B-mediated translocation of the N-terminal portion (fragment A; M_r 22000) into the cell cytoplasm where A inhibits protein synthesis by catalytic ADP-ribosylation of elongation factor 2 (reviews [3,4]).

Diphtheria toxin is able to form pores (or transmembrane channels) in planar lipid bilayers [2], a property attributable to the N-terminal M_r 23000 portion of its fragment B [5]. The pore formation depends drastically on the pH in the protein (peptide)-containing compartment and is maximal at low pH (~4.7) [2,5], as is the rate of entry of diphtheria toxin (or its fragment A) into cells [6–8]. The same low pH induces a change in the conformation of fragment B [8] that exposes a hydrophobic domain located in its N-terminal M_r 23000 region [9]. By amino acid sequence analysis, such a hydrophobic domain has indeed been identified that strongly resembles the transverse lipid-associating domain of intrinsic membrane proteins [1,10]. This domain is also located in the N-terminal region of CB1, the cyanogen bromide peptide (M_r 13000) of fragment B that covers its middle region [1] (fig.1) and is probably responsible for the property of CB1 to induce a strong voltage-dependent increase in ionic conductance across a planar glycerol monooleate bilayer, reflecting the insertion of this peptide into the lipid core

° To whom correspondence should be addressed

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[11]. Here, we present evidence that the capability of CB1 to induce the formation of pores in planar lipid bilayers is strongly enhanced by acidic pH, suggesting that the predicted transverse lipid-associating domain located in the middle region of fragment B might play an important role in the transport of fragment A from acidic endosomes into the cell cytoplasm. We also propose a relationship between the pH dependency of pore formation and the presence of a cluster of prolines in the C-terminal region of CB1.

2. MATERIALS AND METHODS

Diphtheria toxin was obtained from Connaught Laboratories (Willowdale ON) and purified as in [12]. Fragments A and B, and the cyanogen bromide peptides of fragment B were prepared according to published procedures [12]. The purity of protein and peptides was assessed by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate [13] and urea for peptides [14] and by automated Edman degradation [15].

Glycerol monooleate (GMO) was a Sigma product (Sigma Chemie GmbH, München). *N*-Decane was redistilled before use. Planar lipid bilayers were formed from a solution of GMO dissolved in decane (10 mg/ml) on a 1.3 mm diam. aperture in a Teflon cell separating two aqueous phases (3.5 ml) [16,17]. Membrane conductances (G_m) were determined by measuring the specific current (I_m/cm^2) as a function of an imposed potential difference (V_m). Membrane current was measured using Ag/AgCl electrodes connected with a Keithley electrometer (model 602c) as amplifier, followed by a rapid chart recorder. Voltage was monitored with a Keithley microvoltmeter (model 153).

In steady-state experiments, bilayers were formed in the presence of diphtheria toxin or its fragments on both sides and studied either in 10 mM acetate buffer (pH 4.2), 0.15 M NaCl or in 10 mM Tris–HCl buffer (pH 7.2), 0.15 M NaCl. In the pore formation experiments, CB1 was added to one of the compartments (defined as being at ground potential; final conc. CB1 = 1–5 ng/ml) and negative voltage was applied to the other compartment. Bilayers were formed and studied in 10 mM phosphate–citrate buffer at pH 4.2 or 7.2, 1 M NaCl.

3. RESULTS

3.1. *Membrane conductance*

At pH 7.2, the effects induced by fragment B and CB1 (final conc. 10^{-8} M) on the ionic conductance of a planar GMO bilayer (table 1) were about the same as those observed at pH 7.8 [11]: a 5-fold increase in conductance with fragment B and a 19-fold increase with CB1. Diphtheria toxin, fragment A and the other cyanogen bromide peptides of fragment B (final conc. 10^{-8} M) were without effect on the membrane conductance.

When the pH of the aqueous phase bathing the planar GMO bilayer was lowered to pH 4.2, fragment B and diphtheria toxin induced, a 11-fold increase in conductance. The conductance increase was even stronger for CB1, since this peptide induced a dramatic 60-fold increase in membrane conductance. That this effect of diphtheria toxin, fragment B and CB1 was specific is amply demonstrated by the fact that fragment A and the other cyanogen bromide peptides of fragment B did not at all modify the conductance of the planar GMO bilayer (table 1).

3.2. *CB1 forms transmembrane channels*

CB1 contains in its N-terminal region the

Table 1

Effects of diphtheria toxin, fragments A and B, and the cyanogen bromide peptides of fragment B on the conductance of a GMO planar lipid bilayer

Protein or peptide	Conductance ($\times 10^8$ mho/cm^2)	
	pH 7.2	pH 4.2
None	0.38 ± 0.10	0.42 ± 0.10
Diphtheria toxin	0.57 ± 0.12	4.42 ± 0.65
Fragment A	0.40 ± 0.10	0.45 ± 0.12
Fragment B	2.02 ± 0.42	4.60 ± 0.80
CB_1	7.29 ± 0.90	24.80 ± 1.70
CB_2	0.50 ± 0.10	0.50 ± 0.12
CB_3	0.44 ± 0.10	0.50 ± 0.11
CB_4	0.48 ± 0.14	0.45 ± 0.10
CB_5	0.52 ± 0.11	0.45 ± 0.15

Final concentrations of protein, fragments or peptides was 10^{-8} M/l in both compartments. Membranes were formed at pH 7.2 (10 mM Tris–HCl buffer, 0.15 M NaCl) and 4.2 (10 mM acetate buffer, 0.15 M NaCl). Results are the mean of 6–10 expt ± SD

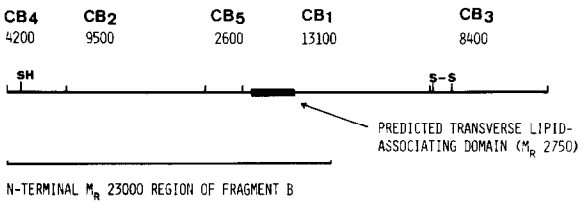

Fig.1. Scale diagram of diphtheria toxin fragment B showing the dimensions and the positions of the predicted transverse lipid-associating domain (heavy line), the cyanogen bromide peptides (CB1 ⟶ CB5) and the N-terminal M_r 23000 region (B45) [9].

predicted hydrophobic transverse lipid-associating domain included in the N-terminal M_r 23000 region of fragment B (fig.1) that has been shown to form transmembrane channels in lipid bilayer at low pH [5]. Therefore, the observed strong effect of CB1 on the conductance of a planar lipid bilayer prompted us to measure the pore formation ability of CB1 at low pH. CB1 was added to one compartment (final conc. 100 ng/ml) and a potential of −40 mV was applied (fig.2). Positive current was defined as positive ions moving from the CB1-free compartment to the CB1-containing compartment. The conductance increased rapidly in steps and finally reached higher values where steps can no more be seen. The shape of the current variation curve looked very similar to that in [2] in rather similar experimental conditions (pH 4.85) for the whole toxin molecule. In order to reveal individual channel conductance, CB1 was added to final conc. 1 ng/ml (77 pM; fig.3, upper panel). After a lag period of about 30–60 s, the conductance in-

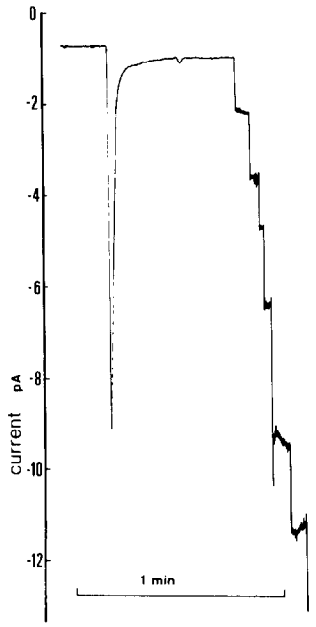

Fig.2. Development of conductance changes in planar lipid bilayer at 100 ng CB1/ml. A −40 mV step was applied across the membrane. CB1 was added in the grounded compartment before the voltage jump (capacitive transcient at the beginning of the record). The bathing solution was 10 mM phosphate–citrate buffer (pH 4.2), 1.0 mM NaCl.

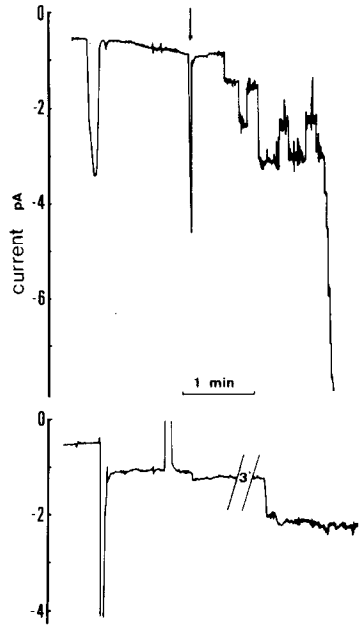

Fig.3. Single channels of cyanogen bromide peptide CB1. Upper panel: a potential of −40 mV was applied before membrane formation, a capacitive current is observed during formation. CB1 (1 ng/ml) was added in the grounded compartment 1 min after membrane formation (arrow). Thereafter, the current increased by step of 0.8 pA corresponding to conductance changes of 20 pS. The bathing solution was 10 mM phosphate–citrate buffer (pH 4.2), 1.0 M NaCl. Lower: the initial jump in current was due to the application of −40 mV potential to the membrane formed at pH 7.2 (same bathing solution as above except for the pH). CB1 (1 ng/ml) was added after 1 min (artefact in positive current) and one pore was observed after 4–5 min with a step of 0.8–0.9 pA, corresponding to a conductance change of 20–22 pS.

creased in discrete step of 0.8 pA corresponding to conductance change of 20 pS identical to the conductance change in [2].

In a series of control experiments at pH 7.2 with 1–10 ng CB1/ml (77–770 pM), the conductance did not change over 15 min except in 2 expt where one step in current was observed respectively after 5 and 7 min (fig.3, lower panel); the current step (0.8 pA) corresponded also to a conductance change of 20 pS. Thus, like diphtheria toxin and the N-terminal M_r 23000 region of fragment B, CB1 shows a strong single-channel activity at low pH; the single channel-conductance measured for CB1 at pH 4.2 is the same as that obtained with diphtheria toxin at pH 4.8 [2] and very similar to that observed with the N-terminal M_r 23000 region of fragment B in similar conditions (pH 5.5) [5].

4. DISCUSSION

These data show that low pH (4.2) strongly enhances the capability of one of the cyanogen bromide peptides of diphtheria toxin fragment B, CB1 to increase the conductance of a planar lipid bilayer and that this capability is associated to an increase of the number of transmembrane channels. Only one other portion of diphtheria toxin has been shown to possess single-channel activity in similar experimental conditions; i.e., the N-terminal M_r 23000 region of fragment B [5]. This region and CB1 share a common amino acid sequence of about M_r 6300 (fig.1) which is, in fact, the N-terminus of CB1. In this common segment is located the previously identified hydrophobic 25 amino acid residues domain similar to the transverse lipid-associating domain of intrinsic membrane proteins [1], characterized by an amphipathic α-helical structure and, in this conformation, a length of 37.5 Å corresponding to the thickness of the bilayer apolar phase. Furthermore, CB4, CB2 and CB5, the other cyanogen bromide peptides included in the N-terminal M_r 23000 region of fragment B have absolutely no influence on the membrane conductance, even at low pH. Therefore, we propose that the capability of diphtheria toxin to form transmembrane channels in lipid bilayers at low pH could well be closely associated to the presence, in the middle of the fragment B molecule, of this particular hydrophobic domain.

At pH 7.2, the hydrophobic domain remains cryptic in diphtheria toxin but is exposed, to different degrees, in fragment B and in CB1. Lowering the pH causes a change in the conformation of fragment B [8] which enhances the accessibility of the hydrophobic domain, accessibility which is now the same in the diphtheria toxin and fragment B molecules, since at low pH, both induce the same membrane conductance. Low pH should also strongly affect the conformation of CB1 which strongly increases, on the membrane conductance, the effect it already has at neutral pH. A careful examination of the almost complete amino acid sequence of CB1 [10,11] reveals, in its C-terminal region, a cluster of 4 prolyl residues embedded in a strongly hydrophobic 14 residues segment (polarity index [18] = 7%):

–Pro–Leu–Pro–Ile–Ala–Gly–Val–Leu–Leu–Pro–Thr–Ile–Pro–Gly–

It has been demonstrated that low pH induces a *cis–trans* proline isomerization process in the case of polyproline [19] and, on the other hand, the conformation of a short hydrophobic peptide (Boc-Pro–Leu–Val-*O*-Me) in hydrophilic solvents differs from its structure, in the apolar phase of a model membrane in the proportion of *cis–trans* isomers of its prolyl residue [20]. Such a modulation of protein conformation by the distribution of proline *cis–trans* isomers has been held responsible for the interaction of immunoglobulins and lipid bilayers at low pH [21]. Therefore low pH could induce in the proline cluster of CB1 a *trans*-conformation leading to the anchoring of diphtheria toxin into phospholipid bilayer membrane, and formation of transmembrane channel with the CB1 hydrophobic domain.

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