

## Chemical and Photochemical Modification of Colicin E1 and Gramicidin A in Bilayer Lipid Membranes

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**Abstract.** Chemical modification and photodynamic treatment of the colicin E1 channel-forming domain (P178) in vesicular and planar bilayer lipid membranes (BLMs) was used to elucidate the role of tryptophan residues in colicin E1 channel activity. Modification of colicin tryptophan residues by N-bromosuccinimide (NBS), as judged by the loss of tryptophan fluorescence, resulted in complete suppression of wild-type P178 channel activity in BLMs formed from fully saturated (diphytanoyl) phospholipids, both at the macroscopic-current and single-channel levels. The similar effect on both the tryptophan fluorescence and the electric current across BLM was observed also after NBS treatment of gramicidin channels. Of the single-tryptophan P178 mutants studied, W460 showed the highest sensitivity to NBS treatment, pointing to the importance of the water-exposed Trp460 in colicin channel activity. In line with previous work, the photodynamic treatment (illumination with visible light in the presence of a photosensitizer) led to suppression of P178 channel activity in diphytanoyl-phospholipid membranes concomitant with the damage to tryptophan residues detected here by a decrease in tryptophan fluorescence. The present work revealed novel effects: activation of P178 channels as a result of both NBS and photodynamic treatments was observed with BLMs formed from unsaturated (dioleoyl) phospholipids. These phenomena are ascribed to the effect of oxidative

modification of double-bond-containing lipids on P178 channel formation. The pronounced stimulation of the colicin-mediated ionic current observed after both pretreatment with NBS and sensitized photomodification of the BLMs support the idea that distortion of membrane structure can facilitate channel formation.

**Key words:** Bilayer lipid membrane — Ion channel — Colicin E1 — Gramicidin A — Tryptophan — Fluorescence — Photosensitizer

### Introduction

Much evidence has been obtained that aromatic residues play an essential role in functioning of membrane proteins and membrane-active peptides, which is apparently associated with the preferential location of these amino acids near the boundary between the hydrophilic and hydrophobic regions of the lipid bilayer (Deisenhofer & Michel, 1989; Schiffer, Chang & Stevens, 1992; White & Wimley, 1998; Epan & Vogel, 1999; Wallace & Janes, 1999; Popot & Engelmann, 2000; Ridder et al., 2000). In particular, tryptophan and other aromatic residues have been shown to provide membrane anchoring (Bhakdi et al., 1996; Vecsey-Semjen et al., 1997; Malovrh et al., 2000; Montoya & Gouaux, 2003), assembly (Srinivasan et al., 1999), formation and stability (Stankowski et al., 1991; Cowan et al., 1992; Weiss & Schulz, 1992; Kreuzsch et al., 1994; Pautsch & Schulz, 1998; Tieleman et al., 1998; Korchev et al., 1998; Campos et al., 1999; Raja et al., 1999; Verza & Bakas, 2000; Yew & Khoo, 2000; Ranatunga et al., 2001) of ion channels and/or regulation of channel conductance either through dipole effects (Becker et al., 1991;

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**Abbreviations:** AIPcS<sub>3</sub>, aluminum trisulfophthalocyanine; BLM, bilayer lipid membrane; DOPC, dioleoylphosphatidylcholine; DOPG, dioleoylphosphatidyl-glycerol; DPhPG, diphytanoylphosphatidylglycerol; DPhPC, diphytanoylphosphatidylcholine; gA, gramicidin A; NBS, N-bromosuccinimide

Killian, 1992; Woolley & Wallace, 1992; Busath, 1993; Hu & Cross, 1995; Koeppe & Andersen, 1996; Sham et al., 2003) or via influence on ligand or substrate binding (Galzi et al., 1991; Schirmer et al., 1995; Yan et al., 1999; Van Gelder et al., 2002).

Studies performed with tryptophan-to-phenylalanine-substituted analogues of gramicidin A (Straessle & Stark, 1992) and single-tryptophan mutants of colicin E1 (Rokitskaya et al., 2001) have indicated that tryptophan residues are responsible for the sensitized photoinactivation of these channel-forming proteins. The photodynamic damage to gramicidin A (gA), a 15-amino acid peptide that has four tryptophan residues at the C-terminus (at 9, 11, 13 and 15th positions) and dimerizes head-to-head to form channels in lipid bilayers (Busath, 1993; Koeppe & Andersen, 1996), has been well documented. In particular, it has been shown that photodynamic suppression of gA-mediated conductance of bilayer lipid membranes (BLMs) is due to a reduction of the number of open channels (Rokitskaya, Antonenko & Kotoro, 1993; Kunz et al., 1995), and leads to fragmentation of the peptide chains at the positions of tryptophan residues (Kunz et al., 1995).

Colicin E1 belongs to a group of water-soluble proteins that can interact with lipid membranes, leading to conformational rearrangements, including formation of an extended two-dimensional helical array on the membrane surface (Zakharov et al., 1998; 1999) that results in creation of ion-conducting pores in lipid bilayers (Cramer, et al., 1995; Gouaux, 1997; Jakes, Kienker & Finkelstein, 1999; Lakey & Slatin, 2001; Zakharov & Cramer, 2002a). The channel-forming domain of colicin E1 contains three tryptophan residues (W424, W460 and W495), whose role in channel formation remains obscure. It should be mentioned that W460 is highly conserved in pore-forming colicins (Lazdunski et al., 1988; Cramer et al., 1995). Recently, while studying sensitized photoinactivation of colicin E1 (Rokitskaya et al., 2001), we have arrived at the conclusion that an aromatic residue in the 495 position is required to reside near a water-membrane interface for the channel formation. To gain further insight into the importance of tryptophan residues for colicin E1 channel activity, we studied here the functional consequences of their chemical and photochemical modification in comparison with the damage to tryptophan residues in gA that occurs in the course of the photodynamic and chemical inactivation of gramicidin channels.

## Materials and Methods

### MUTANTS

"Single Trp" or Trp-less mutants of colicin E1 were prepared by replacing pertinent Trp residues (Trp424, Trp460, and/or Trp495) with Phe (Merrill & Cramer, 1990; Steer & Merrill, 1994). The 178-

residue C-terminal colicin E1 channel polypeptide, P178, was prepared by thermolysin proteolysis of intact colicin E1 (Bishop et al., 1985).

### LIPOSOMES

Liposomes were prepared by evaporation under a stream of nitrogen of a 2% solution of a 70/30 (mol/mol) mixture of diphytanoylphosphatidylcholine (DPhPC, Avanti Polar Lipids, Alabaster, AL) and diphytanoylphosphatidylglycerol (DPhPG, Avanti Polar Lipids) in chloroform (Merck, Darmstadt, Germany), followed by hydration with the buffer solution (pH 4.0) of 120 mM KCl (Fluka, Buchs, Switzerland), 20 mM  $\text{KH}_2\text{PO}_4$ , vortexing and extrusion with an Avanti Mini-Extruder using 0.1  $\mu\text{m}$  pore size Nucleopore polycarbonate membranes. For preparing tryptophan-loaded liposomes, the buffer containing 10 mM tryptophan was used in this procedure. The unbound tryptophan was then removed by passage through a Sephadex G-50 column. Gramicidin A (Fluka) was added from a stock solution in ethanol to the lipid mixture at a concentration of 1% (mol/mol) before evaporation. Colicin (P178) was added from a stock water solution to the buffer solution in the spectrophotometric cuvette. Aluminum trisulfophthalocyanine ( $\text{AlPcS}_3$ ) from Porphyrin Products (Logan, UT) was added to the buffer solution containing liposomes in the spectrophotometric cuvette. Absorption spectra were recorded with an Aminco DW2000 spectrophotometer. Fluorescence was recorded with a Hitachi F-4000 (Tokyo, Japan) fluorescence spectrophotometer. A halogen lamp ("Novaflex", World Precision Instruments, Sarasota, FL), providing an incident power density of 30  $\text{mW}/\text{cm}^2$  was used for illumination.

### BILAYERS

Planar bilayer lipid membranes (BLMs) were formed from a 2% solution of DPhPC/DPhPG (70/30 mol %) or DOPC/DOPG (70/30 mol %) in *n*-decane (Merck) by the brush technique (Mueller et al., 1963) on a 0.55-mm diameter hole in a Teflon partition separating two compartments of a cell containing aqueous solutions of 120 mM (unless otherwise stated) KCl, 20 mM  $\text{KH}_2\text{PO}_4$  at pH 4.0. In single-channel experiments, a cell with a 0.15-mm diameter hole in the teflon partition was used and BLMs were formed from a 2% solution of pure DPhPC. The electric current (*I*) was recorded under voltage-clamp conditions. The currents were measured by means of a patch-clamp amplifier (model BC-525C, Warner Instruments, Hamden, CT), digitized by a LabPC 1200 (National Instruments, Austin, TX) and analyzed using a personal computer with the help of WinWCP Strathclyde Electrophysiology Software designed by J. Dempster (University of Strathclyde, UK). For measurements of the macroscopic currents using a U5-11 amplifier (Moscow, Russia), a voltage of 60 mV (unless otherwise stated) was applied to BLM with Ag-AgCl electrodes placed directly into the cell. In single-channel experiments the applied voltage was 65 mV. The lamp providing illumination was placed at the *cis*-side of the planar membrane. Gramicidin A was added from a stock solution in ethanol to the bathing solutions at both sides of the planar BLM. Colicin (P178) and its mutants were added to the *cis*-side of the membrane. Aluminum trisulfophthalocyanine ( $\text{AlPcS}_3$ ) was added to the *trans*-side of the BLM.

N-bromosuccinimide (Sigma, St. Louis, MO) was added from freshly prepared stock water solutions to the buffer solution containing liposomes in the spectrophotometric cuvette or to the bathing solutions at both sides of the BLM. All experiments were carried out at room temperature (24–26°C).

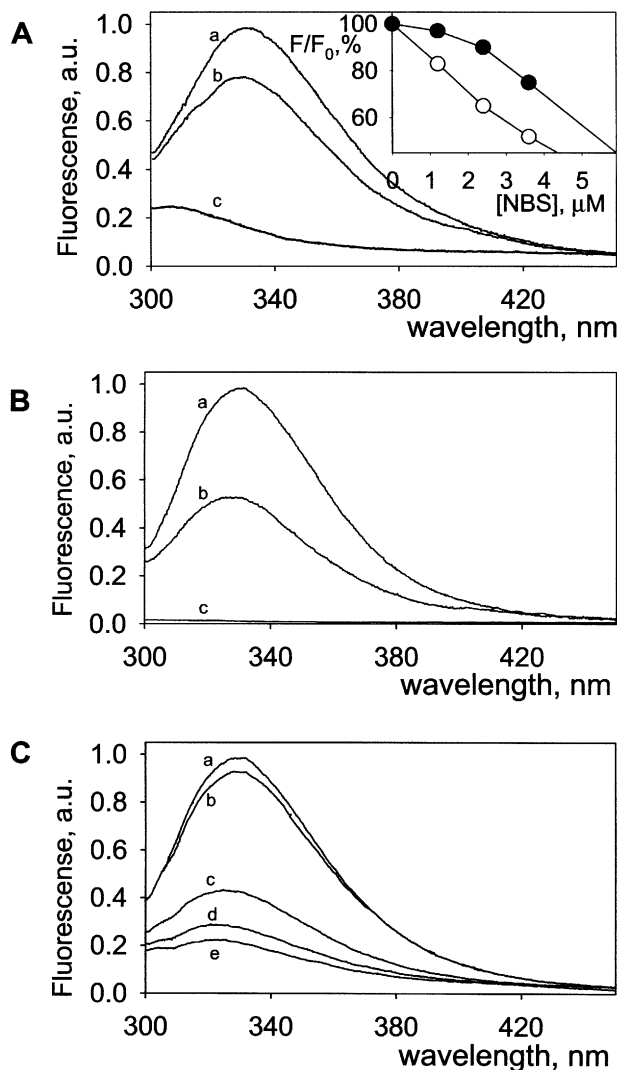
## Results

Figure 1 demonstrates fluorescence spectra of the buffer solutions containing the channel-forming domain of colicin E1 (P178, WT) in the presence (A) and in the absence (B) of DPhPC/DPhPG liposomes before and after addition of N-bromosuccinimide (NBS), the agent known to modify specifically tryptophan residues (Savige & Fontana, 1977; Lundblad & Noyes, 1985). It is seen that treatment with NBS led to a reduction of the tryptophan fluorescence of colicin E1, with higher sensitivity being observed without liposomes. The insert to Fig. 1A presents the concentration dependences of the NBS effect on the fluorescence of P178 either in the presence (filled circles) or absence (empty circles) of liposomes. The colicin fluorescence was suppressed also after exposure of the solution to visible light irradiation in the presence of aluminum phthalocyanine (AlPcS<sub>3</sub>), a potent photosensitizer (Fig. 1C). Prolonged illumination led to almost complete abolition of tryptophan fluorescence. The light-induced decrease in the fluorescence was not detected without the photosensitizer. The addition of singlet oxygen quencher, sodium azide, substantially reduced the AlPcS<sub>3</sub>-mediated effect of illumination on the fluorescence (data not shown). It is worth noting that the photosensitized decrease in the colicin fluorescence was not observed in the absence of liposomes. This result supports the conclusion made previously (Rokitskaya et al., 2000) that photosensitizer binding to lipid bilayers is a prerequisite for effective sensitization.

To ascertain the ability of NBS to permeate through the membrane, we performed experiments with tryptophan-loaded liposomes. The addition of NBS to these liposomes in the tryptophan-free buffer brought about an immediate decrease in the tryptophan fluorescence (data not shown). These data enable us to conclude that the membrane is permeable to NBS.

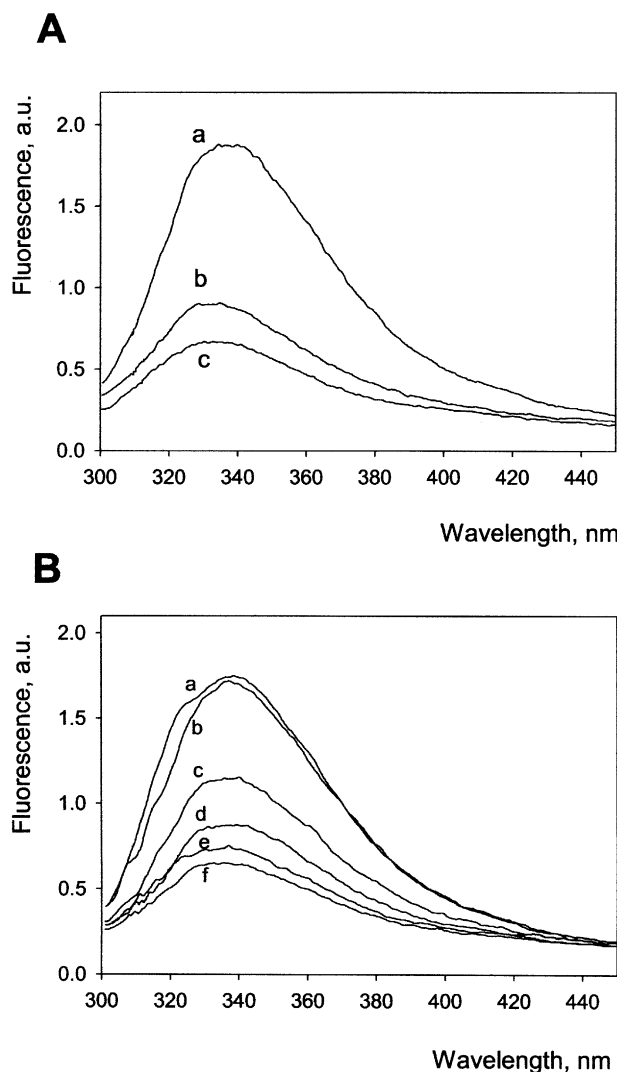
Similar to the effect produced on the colicin E1 fluorescence, NBS added to the buffer solution with liposomes containing gA channels brought about a marked reduction of the gA tryptophan fluorescence (Fig. 2A), in agreement with an early observation made by Boni, Connolly & Kleinfeld, (1986). A dramatic reduction of the gA fluorescence was observed here also upon irradiation with visible light in the presence of AlPcS<sub>3</sub> (Fig. 2B). This effect was substantially diminished upon addition of the singlet oxygen quencher, sodium azide (Lindig & Rodgers, 1981) (data not shown). The photosensitized effect resembles the decline of gA fluorescence after direct excitation of tryptophan residues with UV light (Jones, Hayon & Busath, 1986).

To examine the functional consequences of tryptophan modification, we performed electrophysiological experiments with gA and the colicin E1



**Fig. 1.** Effect of N-bromosuccinimide, NBS (A and B), and photodynamic treatment (C) on the fluorescence emission spectrum of colicin E1 (P178) in the presence (A, C) and absence (B) of liposomes. Curves a are controls in the presence of 0.2  $\mu\text{M}$  P178. Curves b in (A) and (B) are measured after the addition of 3.6  $\mu\text{M}$  NBS. Curves c represent the spectrum of liposomes in the buffer (panel A) and the spectrum of the buffer (B). Insert in (A) is the dependence of the fluorescence intensity of colicin E1, measured at 330 nm, on the concentration of NBS in the presence and in the absence of liposomes (filled and empty circles, respectively). Curve b in (C) is measured after the addition of the photosensitizer, 0.1  $\mu\text{M}$  AlPcS<sub>3</sub>. Curves c, d and e are obtained after successive exposures to 1-min illumination with visible light. The excitation wavelength was 282 nm. The buffer solution contained 20 mM KH<sub>2</sub>P0<sub>4</sub>, 120 mM KCl, pH 4.0.

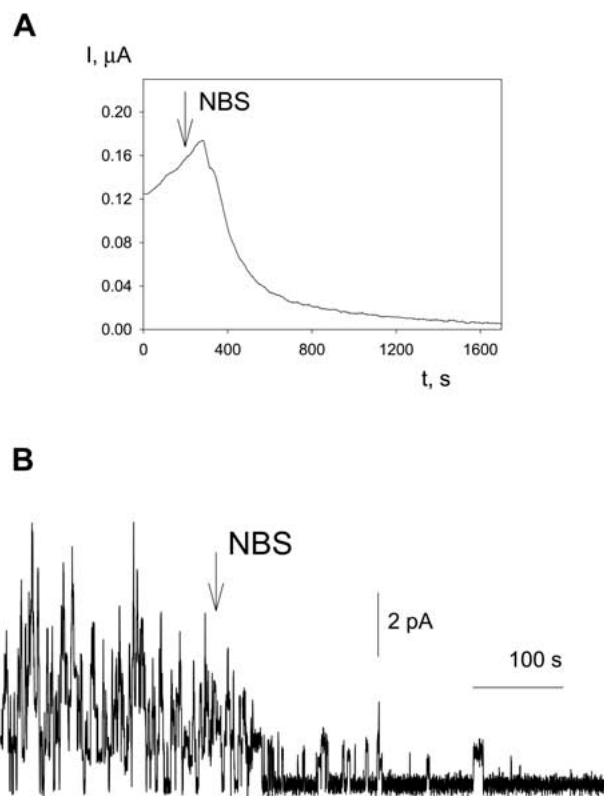
channel-forming domain (P178). Figure 3 shows the effect of NBS on the gA-induced conductance of the planar BLM. It is seen that the addition of NBS to the bathing solutions led to inhibition of the macroscopic conductance, with a delay of about 80 s (Fig. 3A). The recordings made at the single-channel level (Fig. 3B) revealed that the NBS-induced suppression of the gA-induced conductance of the BLM



**Fig. 2.** (A) The changes in the fluorescence emission spectrum of gramicidin A (1.2  $\mu\text{M}$ ) in DPhPC/DPhPG liposomes (curve *a*) induced by the addition of N-bromosuccinimide at a concentration of 24  $\mu\text{M}$  (curve *b*) and 48  $\mu\text{M}$  (curve *c*). (B) The changes in the fluorescence emission spectrum of gramicidin A (1.2  $\mu\text{M}$ ) in DPhPC/DPhPG liposomes (curve *a*) upon the addition of the photosensitizer, 0.1  $\mu\text{M}$  AIPcS<sub>3</sub> (curve *b*), and successive exposures to 1-min illumination with visible light (curves *c*, *d*, *e* and *f*). The buffer solution contained 100 mM KCl, 10 mM Tris, 10 mM MES, pH 7.0. The excitation wavelength was 282 nm.

was mostly due to a decrease in the number of open channels, though some channels with a reduced single-channel conductance were discerned prior to the complete abolition of the gA channel activity. Similar patterns of inhibition were observed previously after photodynamic treatment (Rokitskaya, Antonenko & Kotova, 1993; Kunz et al., 1995), as well as after ultraviolet photolysis (Busath & Waldbillig, 1983; Straessle et al., 1989) and radiolysis (Straessle et al., 1989; Kunz et al., 1995) of gA channels.

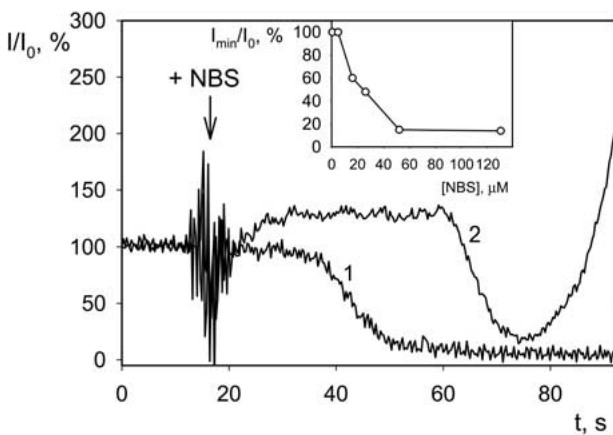
As seen in Fig. 4, addition of NBS to the bathing solutions on both sides of a BLM formed of DPhPC/



**Fig. 3.** The effect of N-bromosuccinimide (NBS) on the current across BLM induced by gramicidin A at the macroscopic (A) and single-channel (B) levels. The BLM was formed from DPhPC. The increase in the current at the beginning of the recording (A) resulted from the continuous incorporation of gramicidin A into the BLM at constant stirring. The addition of NBS (24  $\mu\text{M}$  in A and 100  $\mu\text{M}$  in B) to both sides of the BLM is marked by arrows. The buffer solution was the same as in Fig. 2 and contained 100 mM KCl in A and 1 M KCl in B. The voltage applied to BLM was 30 mV in A and 65 mV in B.

DPhPG led to complete abolition of the macroscopic electric current across BLM mediated by wild type P178 (curve 1). The insert to Fig. 4 shows the concentration dependence of the NBS effect on the colicin-mediated current. To characterize the effect quantitatively, we calculated the ratio of the minimum and the initial current values ( $I_{\text{min}}/I_0$ ). In the case of a DOPC/DOPG membrane, the decrease in the P178-mediated current (the inactivation phase) after the addition of NBS was followed by a steep increase in the current (the activation phase) up to very high levels. Control experiments showed that although NBS sometimes increased the conductance of DOPC/DOPG membranes in the absence of colicin, it was always voltage-independent.

The colicin-mediated current displayed the characteristic voltage dependence (Cramer et al., 1995): it increased rapidly when a *cis*-positive voltage was applied to BLM, and decreased almost to zero level when a negative voltage was applied. It is important that the voltage gating of the colicin-medi-



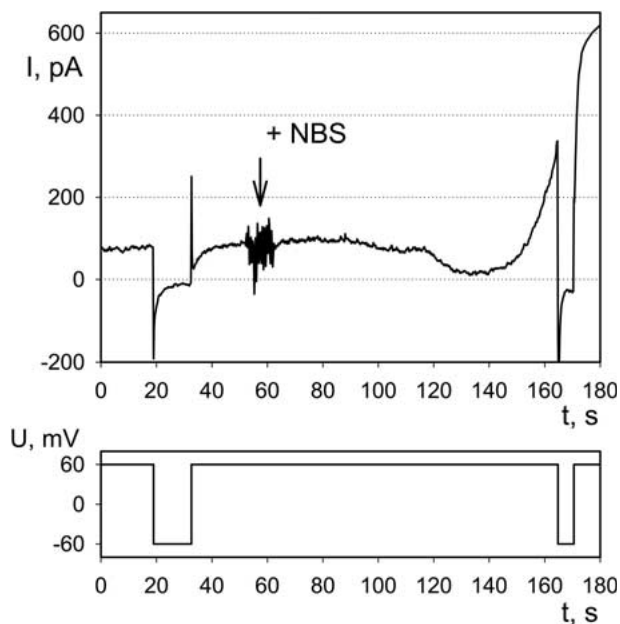
**Fig. 4.** Effect of N-bromosuccinimide (NBS) on the colicin E1 (P178)-induced current across BLM formed from DPhPC/DPhPG (70/30 mol %, curve 1) and DOPC/DOPG (70/30 mol %, curve 2). The addition of NBS (130  $\mu\text{M}$ ) to both sides of the BLM is marked by arrows. The buffer solution was the same as in Fig. 1. One  $\mu\text{g}/\text{ml}$  of colicin E1 (P178) was added at the *cis*-side of the BLM.  $I_0$  was approximately 100 pA. Inset: the ratio of the minimum ( $I_{\min}$ ) and the initial ( $I_0$ ) current values versus the concentration of NBS for the case of the BLM formed from DOPC/DOPG.

ated current was retained after the activation by NBS (Fig. 5). In these experiments, NBS was added on both sides of the membrane. However, the experiments with addition of NBS to one side gave the same results (*data not shown*). This is in agreement with the good permeability of the membrane for NBS shown above.

The effect of NBS on single-tryptophan mutants of colicin E1 was studied only with BLMs formed of DOPC/DOPG, because of very poor incorporation of mutant peptides into DPhPC/DPhPG membranes. Of the mutants studied, only W460 appeared to display the deep inactivation phase upon NBS addition (Fig. 6A,  $I_{\min}/I_0 = 0.25 \pm 0.05$  (Mean  $\pm$  SE)). With W495 (Fig. 6B,  $I_{\min}/I_0 = 0.85 \pm 0.05$ ) and W424 (Fig. 6C,  $I_{\min}/I_0 = 0.8 \pm 0.1$ ), the inactivation by NBS seemed to be negligible.

No inactivation was produced by NBS with the Trp-less mutant (Fig. 6D,  $I_{\min}/I_0 = 1.0 \pm 0.05$ ). It is noteworthy that at higher values of the current induced by W495 and W424 mutants, NBS caused weak but reproducible inactivation.

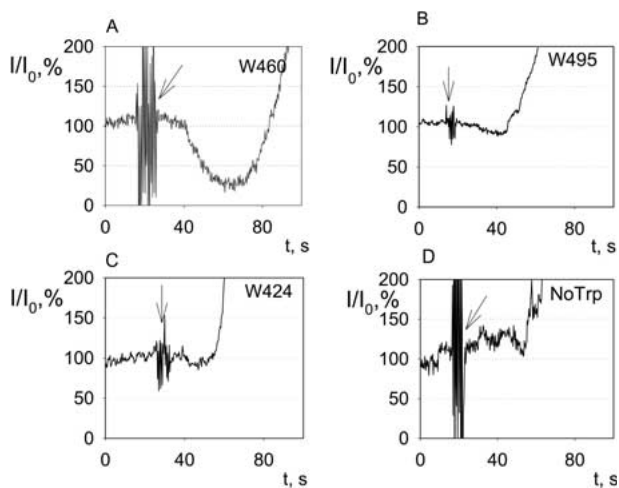
The NBS-induced activation of the current was observed with all the mutant peptides (Fig. 6). Based on these results, it can be assumed that NBS enhances the colicin-mediated current by altering some properties of the lipid bilayer that ultimately facilitate insertion of colicin molecules already bound to the bilayer. To test the validity of this assumption, we measured colicin channel activity in membranes pre-treated with NBS. The action of NBS on the protein was excluded in these experiments because an excess of free tryptophan was added to the mem-



**Fig. 5.** The voltage gating of the colicin-mediated current before and after the addition of NBS (marked by the *arrow*). BLM was formed from DOPC/DOPG (70/30 mol %). Other conditions are the same as in Fig. 4.

brane-bathing solution prior to the addition of the protein. It is seen from Fig. 7 that after the addition of P178 the current across the NBS-pretreated BLM rose to very high values (several nanoamperes), with a short delay of about 30 s. Nevertheless, this high current remained to be voltage-gated (*data not shown*). Thus, pretreatment of BLM with NBS actually promotes colicin insertion. It should be mentioned that prolonged incubation with NBS also increased the background conductance of the BLM, which was always voltage-independent (*data not shown*).

Further investigation of the NBS effect on the colicin E1 channel activity was performed at the single-channel level. As previously shown, colicin E1 forms channels of different single-channel conductance in negatively charged membranes at pH 4 (Cleveland et al., 1983; Bishop et al., 1986; Cramer et al., 1995). These conditions are favorable for the effective binding of the positively charged protein to the membrane surface. However, the concentration of anions, for which colicin E1 has a preferential conductance at pH 4, is significantly reduced near the membrane because of its negative charge. Here we performed single-channel experiments with uncharged (DPhPC) membranes and added excess concentrations of colicin E1 to achieve its insertion. The appearance of the first single-channel openings at a *cis*-positive voltage on BLM was delayed by 50–70 min from the addition of 2  $\mu\text{g}/\text{ml}$  P178 to the bathing solution containing 1 M KCl (Fig. 8). Trace

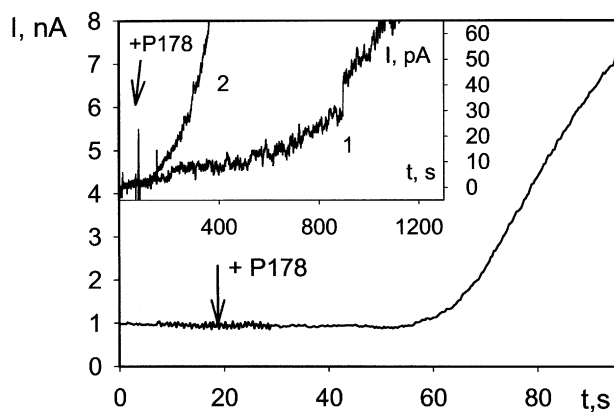


**Fig. 6.** Effect of N-bromosuccinimide (NBS) on the current across BLM induced by colicin E1 mutants. The additions of NBS are marked by arrows. Conditions were the same as in Fig. 4. BLMs were formed from DOPC/DOPG (70/30 mol %).

*A* shows the voltage dependence of the single-channel current induced by colicin E1. It is seen that application of the negative voltage led to shortening of the lifetime and closure of single channels, whereas repeated application of the positive voltage resulted in their opening. The recordings made with uncharged membranes revealed several conductance steps with predominant transitions of 60 pS and 450 pS. The low-conductance steps were characterized by longer average duration than the high-conductance steps (Fig. 8*B*). The presence of negatively charged lipid (DPhPG) in the BLM, even at a very low ratio to DPhPC, led to substantial reduction of the single-channel conductance of both types of channels and a decrease in the single-channel lifetime (Fig. 8*B–E*). For example, at 10% DPhPG, the single-channel conductance of the low-conductance step was decreased 3-fold (Fig. 8*F*). At a higher fractional content of DPhPG, the transitions became too fast and small to be resolved.

It is seen from Fig. 9*A* that the addition of NBS to the bathing solutions at both sides of the BLM formed of pure DPhPC led to suppression of the current fluctuations. Panels *B* and *C* display at better resolution the current traces measured before and after the addition of NBS, respectively. It is seen from these traces that NBS suppressed the low-conductance steps and shortened the open-state lifetime of the 450-pS conductance steps.

An alternative way to modulate the channel activity of colicin E1 is provided by its sensitized photoinactivation, first described by Rokitskaya et al. (2001). Figure 10 illustrates changes in the P178-mediated current across the BLM observed upon irradiation with visible light in the presence of the photosensitizer AIPcS<sub>3</sub>. If the membrane was formed



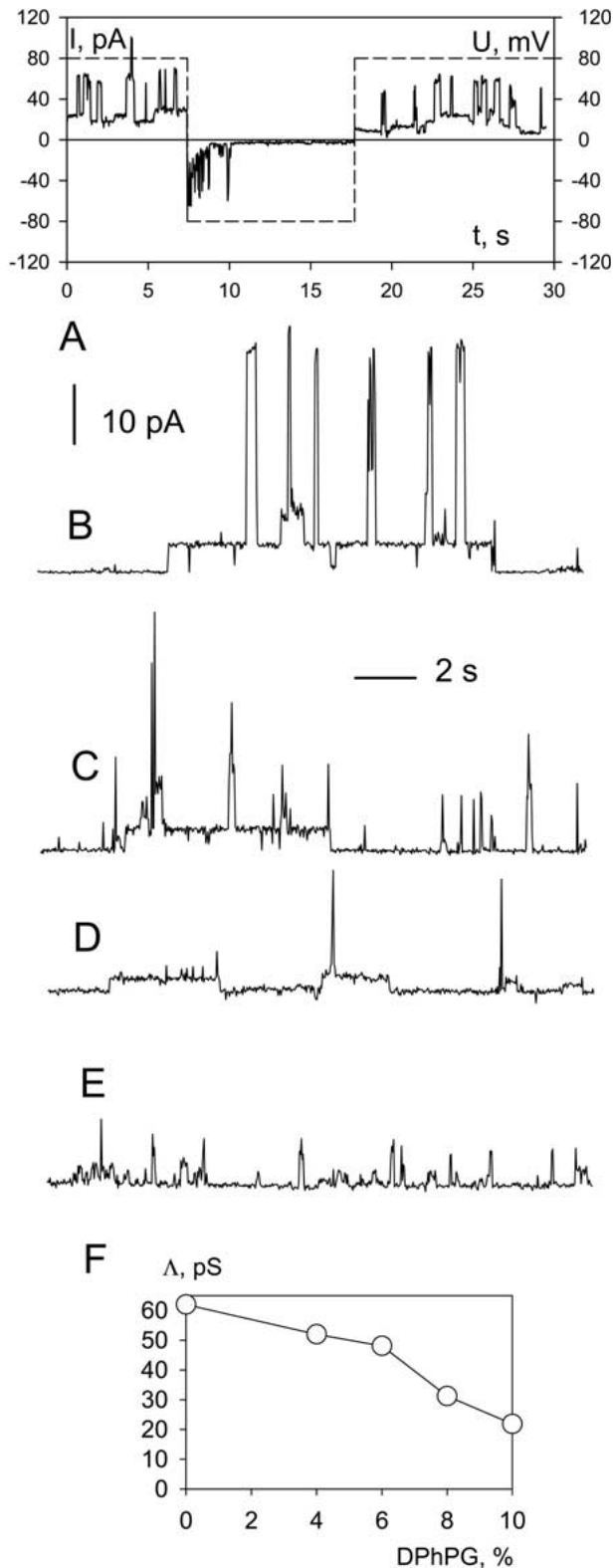
**Fig. 7.** Effect of the pre-treatment of the BLM formed from DOPC/DOPG with NBS (incubation with 130  $\mu$ M NBS for 2 min) on the induction of the current by colicin E1 (P178). The preincubation was terminated by the addition of the excess of tryptophan (2.6 mM). Other conditions were the same as in Fig. 6. Insert: A control experiment with the DOPC/DOPG membrane without the NBS pre-treatment (curve 1). Curve 2 of the insert shows the effect of 100-s pre-illumination of the BLM with visible light in the presence of 1  $\mu$ M AIPcS<sub>3</sub> on the induction of the current by P178.

from DPhPC/DPhPG, only deep inactivation of the current was observed (Fig. 10. curve 1), in agreement with the data of Rokitskaya et al., (2001). A different pattern of photosensitized change in the current was obtained with DOPC/DOPG membranes (curve 2); here, as the illumination proceeded, a small light-induced decrease in the current (the inactivation phase) was followed by pronounced activation. The voltage dependence of the colicin-mediated current was retained after photosensitized activation, as it was after the activation induced by NBS (*data not shown*).

Bearing in mind that photodynamic modification of lipid bilayer structure has been reported to activate some ion channels (Killig & Stark, 2002), we tested the consequences of the photodynamic pretreatment of BLM for the colicin channel activity. As seen from the insert to Fig. 7, pre-illumination of the DOPC/DOPG membrane in the presence of AIPcS<sub>3</sub> for 100 s before the addition of P178 accelerated greatly the induction of the current by colicin E1. Thus, sensitized photomodification of the lipid bilayer stimulated the insertion of colicin channels into the BLM similarly to the NBS treatment of membranes.

## Discussion

The data presented in this paper have demonstrated that the NBS treatment of bilayer lipid membranes containing either colicin E1 or gramicidin A modifies both the functional activity of these channel-formers, as judged by the changes in the ionic current across BLM (Figs. 3, 4), and the intactness of the proteins, as assayed by the loss of tryptophan fluorescence (Figs. 1, 10).



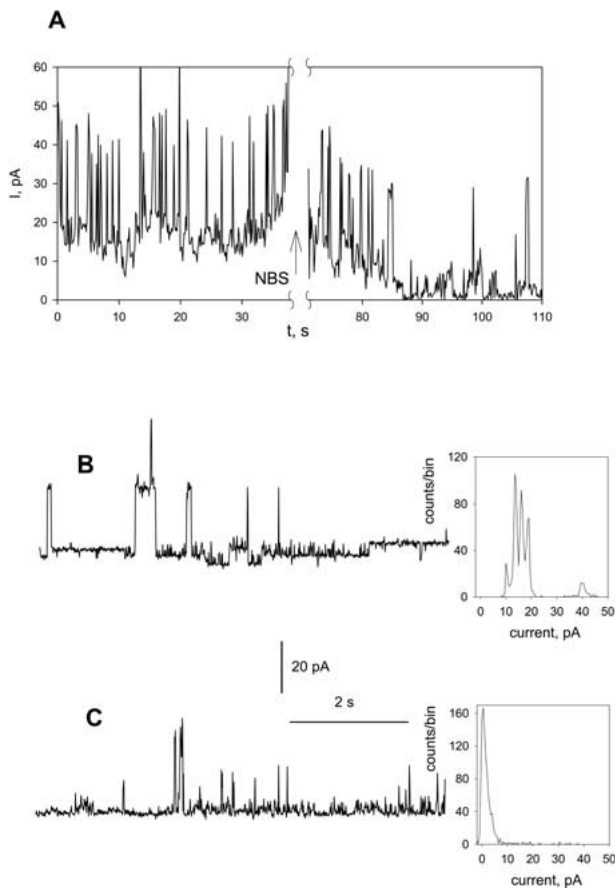
**Fig. 8.** Single-channel traces of colicin E1 (P178) at different contents of DPhPG in the DPhPC membrane (A–E). (A) Changes in the single-channel current upon switching the sign of the voltage applied to BLM. Traces B–E are measured at the BLM voltage of +80 mV. The DPhPG content was varied from 0% (A and B) to 6, 8, and 10% (C, D, and E, respectively). The solution was 10 mM  $\beta$ -alanine, 1 M KCl, pH 4.0. (F) shows the dependence of the single-channel conductance of low-amplitude channels on the DPhPG content.

2). The susceptibility of the fluorescence of colicin E1 in water solution to the NBS effect is in agreement with earlier studies on colicin A (Lakey et al., 1991). It is seen from Fig. 1 that the binding of colicin to liposomes decreases the sensitivity of the protein fluorescence to the action of NBS. It was shown previously that membrane binding of P178 results in its insertion into the membrane interfacial layer (Zakharov et al., 1998; Zakharov & Cramer, 2002b). Reduced sensitivity of P178 tryptophans to NBS can be accounted for by their buried position in the interfacial layer, which decreases their accessibility to the NBS attack. This finding is in agreement with the results of other studies, in particular, those performed with antimicrobial peptides (Schibli et al., 2002).

The effect of NBS on the channel activity of colicin E1 in planar bilayers appeared to depend on the lipid composition of the membrane. In particular, with fully saturated DPhPC/DPhPG membranes only the suppression of the colicin-mediated conductance was observed after the addition of NBS, whereas with DOPC/DOPG membranes having double bonds in the fatty-acyl tail the biphasic pattern of the NBS effect was obtained (Fig. 4). In the latter case, strong activation of the current followed the initial phase of inactivation.

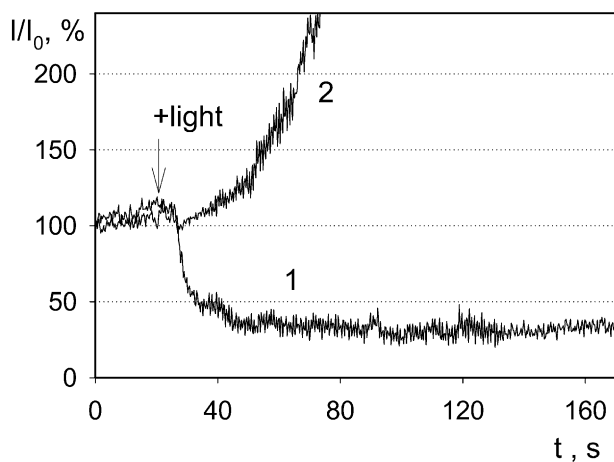
It is reasonable to assume that the NBS-induced inactivation of colicin-mediated current is due to the well-known effect of this agent on tryptophan residues. According to Savage and Fontana (1977) and Lundblad and Noyes (1985), NBS causes conversion of tryptophan to non-fluorescent oxindolylalanine. In most cases the effect of NBS manifests itself in elimination of the functional activity of proteins, implicating the involvement of tryptophan residues in this activity (Spande et al., 1970; Lundblad & Noyes, 1985) though certain examples of activation, in particular of ion channels, were reported (Oxford, Wu & Narahashi, 1978; Cooper, Tomiko & Agnew, 1987). The absence of the inactivation phase in the time course of the current mediated by the Trp-less mutant (Fig. 6D) confirms the relevance of this phase to the effect of NBS on tryptophan residues. Studies performed with all single-tryptophan mutants showed the biphasic pattern of changes in the current after the addition of NBS, with the deepest inactivation being observed with W460. The latter can be ascribed to an essential role of Trp460 in colicin channel functioning, which is supported by the conservation of this residue in pore-forming colicins, as noted in the Introduction. On the other hand, the more pronounced effect of NBS on W460-mediated current can be associated with a greater accessibility of Trp460 to NBS due to the location of this residue at the membrane surface (Suga et al., 1991; Palmer & Merrill, 1994).

The results presented in Figs. 6, 7 suggest that the activation phase is associated with chemical



**Fig. 9.** Effect of NBS (150  $\mu\text{M}$ ) on the single-channel current traces induced by colicin E1 (P178, 2  $\mu\text{g}/\text{ml}$  on the *cis*-side) with the membrane made from pure DPhPC. The solution was 10 mM Tris, 10 mM MES, 10 mM  $\beta$ -alanine, 1 M KCl, pH 4.0. BLM voltage was 65 mV. (B) and (C) preceded and followed (A), respectively. Records in (B) and (C) are accompanied by their current amplitude histograms.

modification of lipid by NBS. NBS has been shown to react with the unsaturated carbon-carbon bonds of fatty acyl groups to form bromohydrins (Carr, Winterbourn & van den Berg, 1996). Other oxidation products not containing bromine, such as dihydroxy derivatives, were detected as well (Carr et al., 1996). Lipid bromohydrins are more polar and bulky than their parent lipids, so they may disrupt both membrane structure and function (Visser, Carr & Chapman, 1998). Taking into account that the specific voltage gating of the colicin-mediated current is retained after its activation by NBS (Fig. 5), it can be concluded that the NBS-induced activation reflects considerable enhancement of the ability of unmodified P178 to form the channel state due to perturbation of membrane structure. These data are in line with the fact that the ability of colicin E1 to induce BLM conductance is sensitive to phospholipid structure: (i) bacterial phosphatidylethanolamine favors formation of the conducting state of colicin E1



**Fig. 10.** Effect of visible light (marked by arrow) on the colicin-induced current across the BLM formed from DPhPC/DPhPG (curve 1) or DOPC/DOPG (curve 2). The photosensitizer (1  $\mu\text{M}$  AlPcS<sub>3</sub>) was added at the *trans*-side. The conditions were the same as in Fig. 4.

more than does DPhPC (Bullock, 1992), although both are electrically neutral; (ii) the addition of very low concentrations of octyl glucoside greatly increased the colicin-mediated BLM conductance (Bullock & Cohen, 1986).

The single-channel measurements (Fig. 9) have revealed that the NBS-induced inactivation of the colicin-mediated current in fully saturated lipid membranes results from a decrease in the number of open channels having a conductance of about 60 pS. It should be mentioned here that only few data (Cleveland et al., 1983; Bishop et al., 1986; Cramer et al., 1995) were reported previously on the single-channel parameters of colicin E1 channels, and none of them were obtained with pure DPhPC membranes. The increased values of the single-channel conductances measured here, as compared to the literature data (ranging from 7 to 60 pS), may be explained by the absence of negatively-charged lipids in our experiments. Actually, negative surface potential created by these lipids should decrease the colicin-mediated current across BLM representing chloride flow at pH 4.0 (Zakharov et al., 1996; 2002). In line with this, the results of Fig. 8 demonstrate the high sensitivity of the colicin single-channel conductance to the presence of negatively charged lipids in the membrane. Importantly, single channels of colicin E1 observed with DPhPC membranes displayed the same voltage dependence (Fig. 8A) as that characteristic of the macroscopic current (Fig. 5).

The results shown in Fig. 10 imply that the photosensitized modification of the colicin channel activity also depends on the nature of the membrane-forming lipid. The photoinactivation of the colicin-mediated current was substantial with DPhPC/DPhPG membranes (*cf.* Rokitskaya et al., 2001 and



Fig. 10, curve 1), and became much smaller, though reproducible with DOPC/DOPG membranes (Fig. 10, curve 2). This is in agreement with the data on sensitized photoinactivation of gramicidin channels (Rokitskaya, Antonenko & Kotova, 1996) and can be ascribed to quenching of singlet oxygen by double bonds. The participation of singlet oxygen in the sensitized colicin photoinactivation was proved previously (Rokitskaya et al., 2001) by the inhibiting effect of sodium azide on this process. Here it is shown that illumination in the presence of the photosensitizer results in azide-sensitive loss of tryptophan fluorescence of colicin E1 and gramicidin A incorporated in liposomes, which supports the conclusion that tryptophan is damaged in the course of the colicin and gramicidin photoinactivation.

The data of Fig. 10 reveal the second phase in the photomodification of the colicin channel activity in DOPC/DOPG membranes, which is seen after the inactivation phase: the pronounced activation of the colicin-mediated current is observed upon further illumination in the presence of the photosensitizer. This activation can be ascribed to promotion of channel formation by unmodified colicin as a result of oxidation of lipid double bonds. This conclusion is supported by the data (see the insert to Fig. 7) showing the activation of colicin channels by the pre-illumination of BLM prior to the addition of the protein.

It can be hypothesized that the dependence of the colicin channel activity on the degree of membrane lipid oxidation is of biological importance. Actually, our data indicate that colicin would preferentially insert into cells with membranes containing oxidized lipids. Since the higher content of oxidized lipids is characteristic of cells that have experienced the oxidative stress, primary attack of these cells by colicin would provide their elimination even at rather low colicin concentrations, which may be beneficial for viability of the cell population as a whole.

As shown in our previous paper (Rokitskaya et al., 2001), among the single-tryptophan mutants of colicin E1, W495 exhibited the highest sensitivity to the photodynamic treatment. In contrast to this, from the data of Fig. 6 it is evident that the highest sensitivity to the NBS treatment belongs to W460. To explain this difference, it should be borne in mind that those tryptophan residues that are exposed to the aqueous phase are known to be most susceptible to the NBS attack (Peterman & Laidler, 1979; Verza & Bakas, 2000; Malovrh et al., 2000), whereas the photodynamic action mediated by singlet oxygen is most effective in a lipid environment due to shortening of the singlet oxygen lifetime in an aqueous medium (Valenzano & Tarr, 1991; Ehrenberg et al., 1998; Krasnovsky, Jr., 1998; Lavi et al., 2002). Therefore, the above data can be well understood within the current views on the colicin E1 channel structure, suggesting Trp495 to be more buried in the

membrane compared to Trp460, which is thought to be located just at the membrane-water interface (Suga et al., 1991; Palmer & Merrill, 1994; Cramer et al., 1995; Malenbaum, Merrill & London, 1998; Tory & Merrill, 1999; 2002). In particular, it is known that Trp495 resides in the hydrophobic hairpin formed by XIII-IX helices that becomes inserted into the membrane at the early stages of the colicin-membrane interaction (Cramer et al., 1995; Elkins et al., 1997; Lindeberg, Zakharov & Cramer, 2000).

In summary, the present results extend our knowledge on the role of different tryptophan residues in the function of colicin E1. In particular, chemical and photochemical oxidation of tryptophan residues led to the suppression of the colicin channel activity, thereby showing the significance of this aromatic acid for colicin channel formation and/or stabilization. On the other hand, oxidation of membrane lipids, caused either by chemical modification or by photosensitization, promoted the transition of unmodified colicin molecules into the channel state, possibly via facilitation of the insertion of amphipathic and hydrophobic alpha-helices into the lipid bilayer.

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