

Reactivation of Streptolysin S by Oligonucleotide

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Oligonucleotide-streptolysin S complex inactivated by alkali treatment remains nonhemolytic, even after acidification and mixing with intact carrier oligonucleotide rich in guanyl residue. Upon dehydration, however, the inactive streptolysin S-oligonucleotide mixture turned to be hemolytic, and this reactivation of the hemolysin was promoted by treatment with guanidine hydrochloride. After alkaline hydrolysis, streptolysin S was freed from nucleotide moiety, by gel filtration through a Sephadex G-50 column. From this nonhemolytic apotoxin as well, active streptolysin S complex was reconstructed upon dehydration with the carrier.

Introduction

Production of active form of streptolysin S (SLS), an oxygen-stable exotoxin of hemolytic streptococci, depends on the presence of certain carrier substance such as yeast RNA [1] or serum components [2]. The toxin is composed of apotoxin peptide and the carrier, and dissociation of the apotoxin-carrier complex results in complete loss of hemolytic activity [2]. Even in the absence of the exogenous carrier, however, streptococcal cells excrete latent SLS which is activable by *in vitro* treatment with carrier (or effector) oligonucleotide [3]. This result implies that active hemolysin can be reconstructed from the free apotoxin and the carrier, under special circumstances. In order to find out conditions for reconstruction of the active hemolysin complex, as well as to elucidate role of the carrier, oligonucleotide-dependent reactivation of SLS has been tested, using alkali-treated nonhemolytic toxin preparation. Subsequently, nucleotide-free SLS has been prepared by passing the alkali-treated toxin through a Sephadex column, and from this nonhemolytic SLS, active hemolysin complex has been reconstructed by dehydration with the effector oligonucleotide.

Materials and Methods

Oligonucleotide and SLS complex

RNase I core of yeast RNA was purchased from Sigma Chemical Co., DNase I and trypsin from Worthington Biochemical Corp., and pronase was a product from Kaken Co., Tokyo. AF (guanylic acid rich oligonucleotide fraction with potent carrier activity for SLS) was prepared from RNase I core of yeast RNA, by DEAE cellulose column chromatography [4]. Oligonucleotide-SLS complex was prepared as described previously [5], and applied on a DEAE cellulose column which had been equilibrated with 0.2 M LiCl in 0.05 M potassium acetate buffer, pH 5.6. After washing with 0.2 M LiCl in the acetate buffer, the toxin complex was eluted with 2 M LiCl in the buffer and collected by precipitation with two volumes of chilled ethanol. After washing with ethanol, the complex was dried *in vacuo*, and preserved at 4 °C. In certain cases, oligonucleotide-SLS complex was pretreated with 10 µg/ml of DNase, in 50 mM Tris·HCl – 2 mM MgCl₂ (pH 7.5) at 37 °C for 2 h and then purified as above. Titration of SLS and definition of hemolytic unit (HU) were as described previously [4, 5].

Treatment with alkali

Oligonucleotide-SLS complex was dissolved in H₂O and pH of the solution was made to 12.5 with KOH. After incubation at 37 °C for 16 h, the solution was neutralized with acetic acid. In certain

Abbreviations: SLS, streptolysin S; HU, hemolytic unit; UV, ultraviolet light.

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cases, oligonucleotide was dissolved in 0.2 M KOH and incubated at 37 °C for 3 h, followed by neutralization with acetic acid.

Guanidine treatment

Each sample was dissolved in 6 M guanidine·HCl and incubated for 10 min at 37 °C or at room temperature. The mixture was diluted 10-fold with 0.1 M KCl and SLS therein was collected by ethanol precipitation and subjected to dehydration.

Dehydration procedure

The sample collected by ethanol precipitation was washed with ethanol and then with ethanol-ether (1:1) mixture. After washing further with ether, the sample was dried in vacuo and preserved in a dessicator.

Others

Gel permeation chromatography of the alkali-treated SLS complex was performed on a Sephadex G-50 superfine column (0.9 × 26 cm), using 2 mM KOH as the eluent. The carrier activity of nucleotide for SLS was determined as described previously [6].

Results

In a preliminary experiment, oligonucleotide-SLS (¹⁴C-labeled) complex was incubated in 0.2 M KOH at 37 °C for 30 min and then passed through a Sephadex G-50 column. The labeled SLS peptide appeared first, whereas elution of the nucleotide was retarded considerably, indicating dissociation of the apotoxin from the carrier. When oligonucleotide-SLS complex (2.3×10^4 HU/ml) was incubated at 37 °C for 16 h at differing pH (adjusted by KOH), and its hemolytic activity was determined after acidification to pH 4.6 with acetic acid, extent of the inactivation reached nearly 90% at pH 11, 98% at pH 11.5 and more than 99% at pH 12. The toxin sample, which had been incubated at pH 12.5 for 16 h at 37 °C and then neutralized, remained non-hemolytic even after mixing with 1 mg/ml of RNase I core. When, however, 2 volumes of ethanol were added to the inactive mixture and the precipitate collected by centrifugation was incubated in 6 M guanidine·HCl and dehydrated, significant fraction (2.5% of the alkali-untreated starting material) turned to be hemolytic. In order to characterize the

reactivation process further, change of hemolytic activity was followed at each treatment step. As shown in Table I, neutralization of the alkali-treated SLS did not restore hemolytic activity. Addition of AF and subsequent acidification to pH 4.6 only slightly activated the hemolysin. On the other hand, the inactive material became significantly hemolytic, upon ethanol precipitation and drying. Further treatment with guanidine·HCl (followed by dehydration) doubled degree of the reactivation. Without supplementation of AF (or RNase I core) to the alkali-exposed toxin sample, yield of the active hemolysin was markedly reduced, regardless of guanidine treatment and dehydration.

During incubation at pH 12.5 at 37 °C for 16 h, the SLS complex probably dissociates into the apotoxin and the carrier oligonucleotide. For hydrolysis of phosphodiester bond, however, the employed condition is insufficient and part of the oligonucleotide molecules may reaggregate upon neutralization and regain the carrier activity. In order to verify this possibility and to obtain the apotoxin devoid of the carrier, the alkali-treated sample was filtered through a Sephadex G-50 superfine column, with 2 mM KOH. As seen in Fig. 1A, the latent SLS activity appeared only in pass-through fractions and, unless activated

Table I. Reactivation of the alkali-treated SLS by interaction with oligonucleotide. Oligonucleotide-SLS complex dissolved in H₂O (Step 1) was incubated at 37 °C for 16 h at pH 12.5 and then neutralized (Step 2). After addition of 10 OD₂₆₀ units/ml of AF and subsequent acidification (Step 3), 2 volumes of ethanol were added to the mixture, and the precipitate was collected and subjected to dehydration (Step 4). The sample was then incubated in 6 M guanidine·HCl at 37 °C for 10 min, precipitated with ethanol, and again dehydrated (Step 5). Aliquot of the sample at Step 2 was acidified to pH 4.6 without mixing with AF, and then subjected to Step 4 and 5 treatments (Step 5a).

Step	treatment	Hemolytic activity [HU/ml]
1	untreated starting material	4.86×10^4
2	incubated at pH 12.5 for 16 h at 37 °C, and neutralized	$< 2.20 \times 10^{-1}$
3	mixed with AF and pH adjusted to 4.6, with acetic acid	1.44×10^0
4	precipitated with ethanol and dehydrated	2.26×10^2
5	treated with guanidine·HCl, precipitated with ethanol and dehydrated	4.48×10^2
5a	treated as Step 5, but in the absence of AF	2.86×10^1

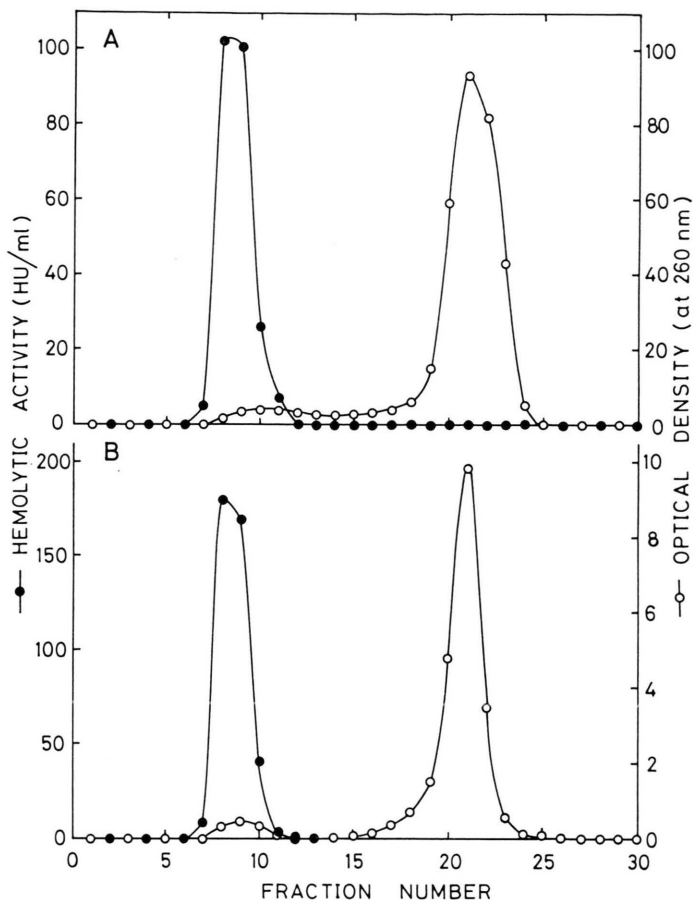


Fig. 1. Dissociation of the SLS complex with alkali. A: Oligonucleotide-SLS complex incubated at pH 12.5 for 16 h at 37 °C was passed through a Sephadex G-50 column with 2 mM KOH, and optical density at 260 nm of each fraction was measured (—○—). After acidification to pH 4.6, RNase I core (2 mg/ml) and KCl (0.1 M) were added to the effluent. Two volumes of ethanol were added to the mixture and precipitate was collected by centrifugation. After dehydration, the sample was treated with guanidine at room temperature for 10 min, diluted with 0.1 M KCl, and subjected to ethanol precipitation. The precipitate was collected, dehydrated again and its hemolytic activity was titrated (—●—). B: Oligonucleotide-SLS complex pretreated with DNase was incubated at pH 12.5 for 16 h at 37 °C and subjected to gel filtration as in A. Latent SLS in the pass-through fractions was collected by ethanol precipitation, incubated further in 0.2 M KOH at 37 °C for 3 h and filtered again through the Sephadex column. Symbols were the same as in A.

by dehydration with RNase I core or AF, each fraction (neutralized with acetic acid) remained entirely nonhemolytic. Reflecting alkali-induced dissociation of the carrier, elution of bulk oligonucleotide was retarded markedly. (Without the alkali treatment, the carrier oligonucleotide is excluded from the Sephadex matrix and eluted within the void-volume.) When the dissociated oligonucleotide was collected by acidic ethanol precipitation and then incubated with resting streptococci, a moderate amount of SLS was produced: its carrier activity (170 HU/OD₂₆₀) was about 30% of the untreated RNase I core.

Although more than 96% of the carrier oligonucleotide was removed by the gel permeation chromatography, the latent SLS fraction contained a detectable amount of UV absorbing substance. In order to eliminate possibility of contamination of DNA, oligonucleotide-SLS complex pretreated with DNase

I was incubated at pH 12.5 for 16 h, at 37 °C. The sample was passed through the Sephadex G-50 column and the fractions activable by dehydration with oligonucleotide were pooled. From the pooled sample, the latent SLS was collected by precipitation with acidic ethanol, incubated further in 0.2 M KOH at 37 °C for 3 h, and then rechromatographed on the Sephadex column. As shown in Fig. 1B, the latent SLS was eluted again in the pass-through fractions, whereas nucleotide moiety was localized in the rear fractions. After the second chromatography, no UV-absorption peak was detected in the latent SLS sample (pooled and neutralized with acetic acid), around 250–270 nm. When the nucleotide-free SLS fraction, totally devoid of hemolytic activity, was dehydrated after treatment with RNase I core and guanidine·HCl, significant level of hemolytic titer was recovered (Table II). Preincubation with trypsin did not affect degree of the reactivation. Unless

Table II. Reactivation of SLS by oligonucleotide. Nucleotide-free SLS was prepared as described in Fig. 1B legend (Step 1). To the SLS fraction, RNase I core (25 OD₂₆₀ units/ml) and KCl (60 mM) were added (Step 2), and mixed with 2 volumes of cold ethanol. The precipitate was collected by centrifugation, dried, and incubated in 6 M guanidine · HCl for 10 min at room temperature. SLS in the mixture was then collected by ethanol precipitation and dehydrated (Step 3). Step 3b treatment was essentially similar to Step 3, except that nucleotide-free SLS fraction preincubated with 10 µg/ml of trypsin at 37 °C for 2 h was used.

Step	treatment	Hemolytic activity [HU/ml]
1	untreated starting material	0
2	mixed with RNase I core	0.1
3	treated with guanidine · HCl and dehydrated after ethanol precipitation	40.0
3a	treated as in Step 3, but in the absence of RNase I core	0.2
3b	preincubated with trypsin, and then treated sequentially as in Step 3	42.8

oligonucleotide (or RNase I core) was added as the effector, hemolytic activity was not restored by guanidine treatment and dehydration. Hemolytic activity of the reactivated SLS was, like that of the native toxin, destroyed by pronase and inhibited by trypan blue.

Discussion

Prolonged incubation of oligonucleotide-SLS at pH 12.5 results in complete loss of its hemolytic activity. The activity is, however, partially restored, when the alkali-treated mixture is dehydrated after acidification and mixing with oligonucleotide or RNase I core. Under the alkaline condition, SLS peptide per se might irreversibly be damaged to some extent, and this possibility might partially be responsible for low recovery of hemolytic activity. For the dehydration-dependent reactivation, addition of oligonucleotide is distinctly effective, but feeble SLS activity is recovered even in the absence of

the added effector. The alkaline condition employed causes dissociation of the SLS complex, but some fraction of the oligonucleotide can still serve as the carrier upon neutralization and dehydration. In order to prevent reassociation, SLS moiety and the bulk oligonucleotide in the alkali-treated sample have been separated by passing through a Sephadex G-50 column, using 2 mM KOH as the eluent. The latent SLS sample eluted in the void volume remains nonhemolytic, even after acidification and dehydration. Although its reactivation absolutely depends on the added oligonucleotide, the latent SLS fraction is associated with a small amount of UV-absorbing substance. Removal of this residual nucleotide moiety is attained only after alkaline hydrolysis in 0.2 M KOH at 37 °C, followed by rechromatography through the Sephadex column.

As expected, the nucleotide-free SLS preparation per se does not exhibit any hemolytic activity. Neither simple incubation with the oligonucleotide nor dehydration in the absence of the effector leads to reactivation of the hemolysin. Like reactivation of the latent SLS obtained from the carrier-free spent medium [3], dehydration with the effector oligonucleotide is essential for reconstruction of the active SLS complex. These results indicate that SLS peptide (apotoxin) freed from its carrier suffers reversible denaturation by distorted hydrogen bonding (and/or hydrophobic interaction) and not by hydrolysis of covalent bond. Probably, hydration of SLS peptide blocks proper interaction with the effector oligonucleotide and/or its receptor on erythrocyte membrane. In neutral aqueous solutions, carrier activity of the oligonucleotide is maintained, whereas inactivation of oligonucleotide-SLS proceeds without dissociation of the complex. Once dried, however, SLS complex is rather stable. In addition, heat-inactivated RNA-SLS preparation is partially reactivable by dehydration [7]. Taken together, dehydration is most probably required for conversion of the latent SLS-oligonucleotide complex into the active conformation.

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