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Streptolysin O

II. Relationship of Sulfyhdryl Groups to Activity

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Streptolysin O (SO), a group A streptococcal toxin, exists in two forms, a reduced active state and an oxidized reversibly inactive state. Activity is measured by red blood cell hemolysis. SO is a labile toxin, and, with time, activity is irreversibly lost. The rate of activity loss is slowed by incubation with 0.1 M 2-mercaptoethanol or 0.01 M ethylenediaminetetraacetic acid (EDTA). The effect of EDTA can be reversed by excess $MgSO_4$. Reversibly oxidized SO is activated by cleavage of disulfide bonds. When the free sulfhydryl groups of the active SO are alkylated with iodoacetamide, complete and irreversible loss of activity results. Periodate (0.01 M) oxidation also causes complete loss of activity which may be due to oxidation of sulfhydryl groups. SO in the active form reacts with Fe^{2+}, Cu^{2+}, Ca^{2+}, and Mg^{2+}, causing loss of activity in various degrees depending on the ions and the concentration used. Ferric and cupric ions are most effective and cause loss of activity at concentrations on the order of 10^{-4} M. The reversibly oxidized form of SO is not influenced by exposure to cupric ions (0.01 M), indicating that the reaction is only with the active form of SO, probably involving the free sulfhydryl groups. These groups may be responsible for the direct binding of the toxin to the target membrane or for the maintenance of the proper conformation for activity.

Streptolysin O (SO), an oxygen- and heat-labile streptococcal hemolysin, had been shown to cause both lysis of cells (2, 3) and cardiotoxicity (7, 11, 12, 15). When SO is secreted by streptococci, it is in an active state, but it rapidly oxidizes to a reversibly inactive state with the formation of disulfide bonds. Reactivation is accomplished by cleavage of these disulfide bonds after exposure to thiol-reducing agents such as cysteine, sodium thioglycolate, or 2-mercaptoethanol (2-ME). The role of the resulting free sulfhydryl groups is not presently known. In attempting to clarify this problem and to find methods of prolonging SO activity, reduction, alkylation, and oxidation, experiments were conducted.

MATERIALS AND METHODS

Partially purified SO was prepared from a 16-hr culture of *Streptococcus pyogenes* group A type 3 Richards strain grown in Todd-Hewitt broth. The bacteria were removed from the culture by continuous-flow centrifugation in a Sorvall centrifuge at room temperature spinning at 12,100 × *g*. Concentration of the supernatant solution was accomplished by freezing at −20 C for 48 hr, thawing at 4 C, and collecting the first fraction of the supernatant to thaw (one-fifth of the total volume). This fraction was then refrozen at −20 C and then thawed once more at 4 C, again the first one-fifth of the sample to thaw being collected. The concentrated broth was then precipitated with 65% saturated ammonium sulfate and centrifuged; the precipitate was dissolved in a minimal amount of phosphate-buffered saline (PBS), *p*H 7.0. A 6-ml amount of the solution was then applied to a 500-ml Sephadex G-100 gel filtration column (25 by 1,000 mm) which had been equilibrated with PBS at 4 C or with 0.001 M barbital, 0.15 M NaCl buffer. Fractions were collected, and the elution peak containing the SO was determined by red blood cell lysis as previously described (16). Protein elution peaks from the column were determined with an LKB Uvicord recorder measuring absorbance at 280 nm. The area under the region of SO was less than 10% of the total area of the absorbancy peaks. Fractions showing activity were pooled and in some cases concentrated by ultracentrifugation in a Beckman-Spinco model L ultracentrifuge at 100,000 × *g* for approximately 22 hr or by the use of acrylamide beads (Lyphogel, Gelman Instrument Co., Ann Arbor, Mich.). All preparations could be completely deactivated by antistreptolysin O, and their biological activity could be increased by reduction and decreased by oxidation. These properties are compatible with SO. In some experiments, a commercial SO preparation was also used (Behring Diagnostics, Woodbury, N.Y.). This preparation is a product of group O streptococci. All of the experiments in this paper were performed with both sources of SO except those involving addition of Fe^{2+} or Cu^{2+}

ions in which only group ASO was used. Data from representative experiments have been included here. In no case did the results differ significantly between the two sources of SO.

SO activity was titrated by the Microtiter technique as described by Van Epps and Andersen (16) with the exception that activation of the SO was accomplished with 0.1 M 2-ME. If hemolysis occurred but a partial button of red blood cells still remained in the last tube of the titration, it was read as one-half of a tube.

RESULTS

Effects of reducing environment on SO activity. Since maintenance of activity is a crucial problem in working with SO, we have tested effects of a strong reducing agent on the persistence of SO activity. To accomplish this, a sample of SO was put in a dialysis sack and dialyzed against 0.1 M 2-ME at room temperature. As a control, another sample was dialyzed against PBS at room temperature. Periodically, SO activity was determined, both with and without 2-ME added as an activator to the titrated sample. The final concentration of 2-ME used to activate the toxin when titering was equivalent to the final concentration used in dialysis. After 1.5 days, the activity resulting from a 20- to 30-min reductive activation of the PBS-dialyzed SO control was equivalent to the activity of the experimental SO accomplished by 1.5 days of dialysis against 0.1 M 2-ME (Fig. 1). Thus, the difference in activity observed later is not due to the length of the incubation time with 2-ME. This is also demonstrated by the lack of an increase in the SO titer with time. These data indicate that SO activity remains active longer when the toxin is kept in a 2-ME environment. After a period of 2 weeks, the titer of the SO kept in this reducing environment still remained essentially the same, whereas the control had lost all activity at the end of 5 days.

Alkylation of SO. The role of the free sulfhydryl groups resulting from reductive activation of SO was studied by alkylation with iodoacetamide

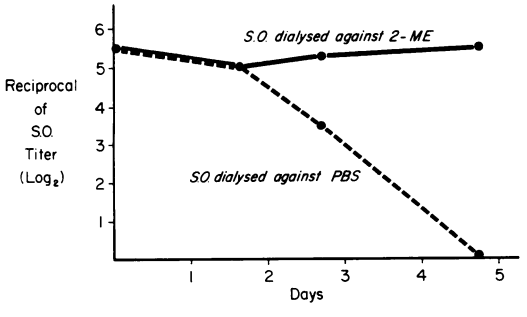

FIG. 1. *Effect of 0.1 M 2-mercaptoethanol on maintenance of streptolysin O activity.*

TABLE 1. *Effect of iodoacetamide alkylation on reduced and oxidized streptolysin O (SO)*

No reducing agent added in titering[a]			Reducing agent added in titering		
SO reduced and not alkylated	SO reversibly oxidized and alkylated	SO reduced and alkylated	SO reduced and not alkylated	SO reversibly oxidized and alkylated	SO reduced and alkylated
4+	±	0	5	4	±

[a] The numbers represent the $\log_2$ of the reciprocal of the SO titer.

(IA). Reduction was accomplished by exposure to 0.1 M 2-ME for 30 min which yields maximum activation (10). Alkylation was performed by the direct mixing of equal parts of 0.6 M IA with the activated SO in PBS. As an SO control, a similar sample of activated SO was used adding water instead of IA. The alkylation control consisted of equal parts of a reversibly inactive preparation and 0.6 M IA. A reaction time of 30 min at room temperature was allowed for all samples. The preparations were dialyzed against PBS to remove both activating agent and IA. The results in Table 1 show that alkylation significantly decreases lysis of red blood cells (RBC) by SO. In each case, SO was titered with and without the addition of a reducing agent for the activation at the time of titering. The purpose of this was to make sure that complete alkylation had taken place. If it had, no reversible disulfide bonds should be present. It also demonstrated that SO in the reversibly oxidized inactive state is not susceptible to the action of IA as evidenced by its reductive activation after exposure to alkylating conditions. Reduction followed by alkylation clearly causes marked loss of SO activity.

Alkylation of red blood cells. Since alkylation of the SO sulfhydryl groups drastically impedes activity, it could be hypothesized that these groups may be responsible for attachment to the cell membrane. One possible target for attachment would be the free sulfhydryl groups of the cell membrane (R. I. Weed and G. G. Berg, Fed. Proc. **22**: 213, 1963). Although this seems unlikely since lysis does occur under reducing conditions, the following experiment was conducted to exclude this possibility. Rabbit RBC were subjected to both strong (equal volume of 0.01 M 2,3-dimercaptopropanol in PBS) and weak (equal volume of 0.005 M solution of cysteine hydrochloride in PBS) reducing conditions. The concentration of reducing agents used was similar to what would be used for SO activation in assaying the toxin. Equal volumes of the cysteine-treated RBC and normal RBC were subjected to alkylat-

ing conditions (4) by dialysis against 20 volumes of 0.32 M IA for 30 min. Those cells treated with 2,3-dimercaptopropanol were not alkylated because a spontaneous lysis occurred when they were dialyzed against IA. These 2,3-dimercaptopropanol-treated cells were used to see whether the resulting free sulfhydryl groups affect SO activity. All cells including those reduced with 2,3-dimercaptopropanol were dialyzed against PBS to remove IA, activating agent, or both. Each preparation of RBC was then made to a 2% solution of PBS and used as a source of cells in a Microtiter assay by using a standard SO preparation. In all cases, the RBC lysed equally, demonstrating that membrane sulfhydryl groups play no role in the SO membrane interaction.

Periodate oxidation of SO. Oxidation with periodate was also carried out by a procedure similar to that of Andersen et al. (1). One milliliter of SO was mixed with 1 ml of 0.02 M sodium metaperiodate in PBS. The solution was incubated at room temperature for 30 min, and then dialyzed overnight against 500 ml of 0.01 M sodium metaperiodate in PBS. The control and experimental solutions were then titered for SO activity with and without the addition of equal volumes of 0.1 M 2-ME in the assay. The results in Table 2 show that oxidation by periodate rapidly and completely causes irreversible inactivation of the SO.

Effects of EDTA, $MgSO_4$, $FeCl_2$, $CuSO_4$, and $CaCl_2$ on streptolysin O. Since metal ions have been involved in protein stabilization or activation and also in reactions with sulfhydryls (9, 14), the effect of removing metal ions from the system was tested. Binding of these ions was accomplished with 0.01 M disodium ethylenediaminetetraacetate (EDTA) as a chelating agent. Testing was done over a period of 2 days, employing two 1.8-ml samples of SO, adding either 0.2 ml of 0.1 M EDTA or 0.2 ml of water to each sample. The preparations were allowed to remain at room temperature and were periodically titered to determine the amount of oxidation which had taken place. The titration was done with and without the addition of reducing agent (0.1 M 2-ME), allowing a reaction time of 20 min. The results of these experiments are shown in Fig. 2. The data demonstrate that EDTA prolonged SO activity as compared to the control. It is possible that the observed effect by EDTA is not due to its chelating capacity but to some other activity. An attempt was made to overcome the effects of EDTA with Mg^{2+}. This should then support or refute the chelating effect of EDTA on the preservation of SO activity. A 4-ml amount of SO was divided into two equal parts and to each was added 0.2 ml of a 0.1 M EDTA solution. The mixture was allowed to incubate for 1 hr at room temperature to assure binding of available cations. Then, 0.4 ml of 0.1 M $MgSO_4$ solution was added to one sample, and 0.4 ml of water to the other. The samples were then titered periodically to measure the amount of inactivation taking place. It is evident in Fig. 3 that the presence of Mg^{2+} in excess of EDTA has increased the rate of inactivation.

TABLE 2. *Effect of periodate oxidation on streptolysin O (SO)*

No reducing agent added in titering[a]		Reducing agent added in titering[a]	
SO control	Periodate oxidized SO	SO control	Periodate oxidized SO
2+	0	4+	0

[a] Numbers represent the $\log_2$ of the reciprocal of the SO titer.

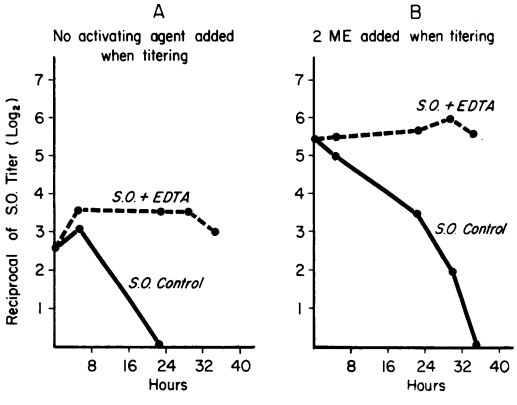

FIG. 2. *Prolonging streptolysin O activity with 0.01 M EDTA.*

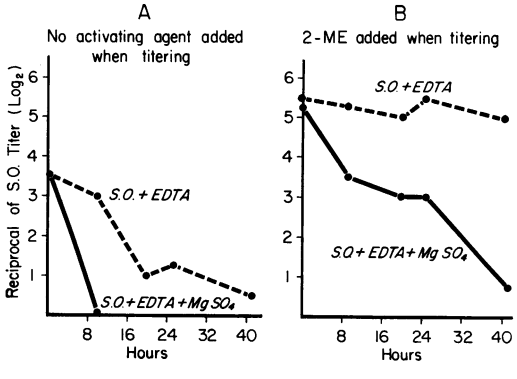

FIG. 3. *Reversal of the EDTA effect on streptolysin O by excess $MgSO_4$.*

Other metal ions were tested to see whether these would react with active SO. To determine whether the active form was affected, preparations of active SO were mixed with various concentrations of $FeCl_2$, $CuSO_4$, $CaCl_2$, $MgSO_4$, and EDTA. To each of the 14 0.9-ml samples of SO, 0.1 ml of an appropriate concentration of each of the metals used was added. The final concentrations are shown in Fig. 4. To the control, 0.1 ml of water was added. All titers were performed in the absence of reducing agents since precipitation with 2-ME occurs with some metals. The preparations were in a 0.001 M barbital, 0.15 M NaCl (*p*H 7) buffer, since precipitation also occurs with metal ions and PBS buffer. The results in Fig. 4 show that metals will react with active SO and cause marked inactivation. Almost total deactivation occurs with $FeCl_2$ and $CuSO_4$, whereas $CaCl_2$ appears to show a graded inactivation which is concentration-dependent. Magnesium does not show an initial effect as expected from the previous graph.

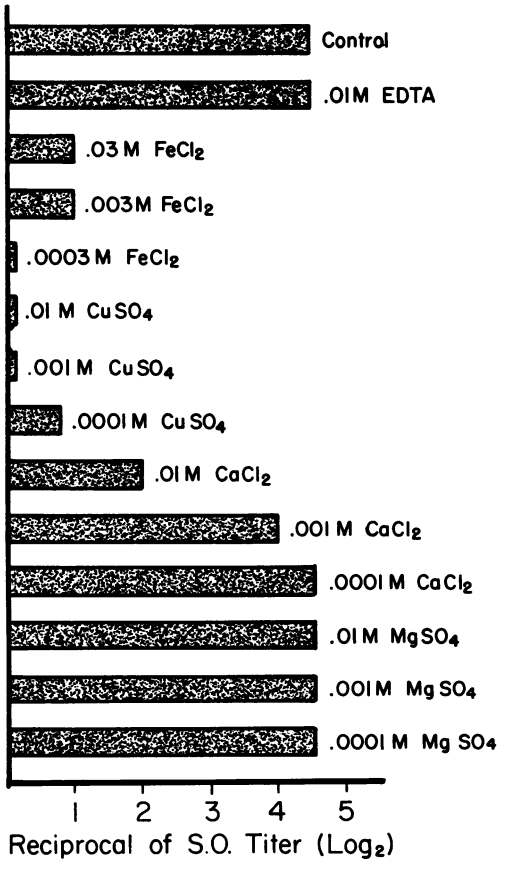

FIG. 4. *Deactivation of streptolysin O by reaction with metal ions.*

The ability of reversibly inactive SO to react with metal ions was determined by utilizing a completely reversibly deactivated SO preparation and a completely active SO preparation, both having titers of 1:4 with activation. Each sample was divided into 2 portions of 0.9 ml each. One active SO sample and one inactive sample were each mixed with 0.1 ml of a 0.1 M $CuSO_4$ solution; the remaining two samples, one active and one reversibly inactive, were each mixed with 0.1 ml of water as control experiments. The preparations were dialyzed against a 0.001 M barbital, 0.15 M NaCl (*p*H 7) buffer. The active SO preparations were then titered, and loss of activity with $CuSO_4$ was shown to occur, as in the previous experiment. However, the reversibly inactive SO preparation showed no loss of activity when reduced. It can be concluded that $CuSO_4$ when reacted with SO in the oxidized state has no effect on the potential SO activity when the toxin is reduced.

DISCUSSION

Cleavage of one or more disulfide bonds in the inactive SO molecule is necessary to permit return of its ability to lyse cell membranes. This activation might be the result either of a conformational change in SO or the availability of free sulfhydryl groups necessary for some reaction in the course of cell lysis. A conformational change could cause activation by exposing or altering a site on the SO molecule capable of interaction with the cell membrane.

Since RBC with alkylated sulfhydryl groups were shown in this study to retain susceptibility to SO lysis, we can exclude the possibility that disulfide bond formation between SO and the cell membrane is necessary for the lytic action of SO. It is possible, however, that sulfhydryl groups on SO react directly with other membrane structures.

Alterations of the free sulfhydryl groups after reduction were performed to help determine the mechanism of activation and action. If free sulfhydryl groups are necessary for the activity, then the blocking agents would decrease activity. Alternatively, if activity depends on preventing the reformation of certain disulfide bonds, then sulfhydryl blocking agents would be expected to maintain SO in a permanently active state. Our observation that IA inactivates SO suggests that sulfhydryl groups are directly involved in the action of SO on the cell membranes. This cannot, however, be taken as proof since the carboxymethylated sulfhydryl groups may cause distortion of the molecule preventing membrane interaction. It is also feasible that if the carboxymethylated group is near the active site, it could cause stearic hindrance and thus interfere with the action of the toxin. Reduction-alkylation pro-

cedures have previously been performed on SO (8, 13), but in each case activity could be restored by reducing agent. In our experiments, loss of activity after alkylation could not be reversed. The results of the earlier experiments can be attributed to failure of the reducing agent employed to activate completely all of the inactive SO prior to alkylation. Thus complete alkylation could not be achieved.

Oxidation by periodate also destroyed SO activity. Its action could be attributed to oxidation of cystine and cysteine residues. This oxidation of sulfhydryl groups to sulfoxides could be expected to cause conformational changes which would deactivate the toxin as similarly speculated for alkylation. Another possibility arises; since periodate oxidation is not as specific for sulfhydryl groups as IA alkylation, the deactivation could be attributed to oxidation of other amino acids or even a carbohydrate moiety if one exists (5, 6).

EDTA and Mg^{2+} have opposing effects on the activity of SO. The ability of EDTA to prolong SO activity seems directly related to the binding of cations, since this effect can be overriden by excess Mg^{2+} (Fig. 3). The effect of Mg^{2+} does not appear to be on the reducing agent since, at the onset of the experiment, SO both with and without Mg^{2+} reached the same titer when the reducing agent was added. It is possible that Mg^{2+} reacts with the free sulfhydryls of the active form of SO rendering it inactive. The data in part A of Fig. 3 show that the SO in the active state at the onset of the experiment was inactivated by Mg^{2+} 10 hr later.

In analyzing the effects of Cu^{2+}, Fe^{2+}, Ca^{2+}, and Mg^{2+} on SO, the data show a direct reaction on the active form of SO. Ferric ions and cupric ions appear to be the most effective since almost complete loss of activity is evident even at concentrations as low as 10^{-4} M. Calcium apparently has a lesser effect, and a definite decrease in deactivating capacity occurs as we approach 10^{-4} M. The significance of these data is strictly qualitative since the SO preparation was not completely pure and the number of competitive groups for metal ions is not known. Magnesium does not have an immediate effect on the loss of activity (Fig. 4), but there is a definite although slower reaction when Mg^{2+} is used in overriding the effects of EDTA (Fig. 3). The failure of metal ions to inhibit the potential activity of SO in the reversibly inactive state suggests an interaction with sulfhydryl groups although other groups exposed by reductive activation may participate.

The importance of free sulfhydryl groups in maintaining the activity of SO is demonstrated by the prolonged activation in the presence of reducing agents, loss of activity by alkylation, and loss of activity by exposure to sulfhydryl-reacting metal ions. The information obtained from these experiments will be useful in analyzing the role of the sulfhydryl group in the SO membrane interaction and also render pertinent information concerning the maintenance of SO activity.

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