(F. E. LaBar, unpublished data). Thus, good results with heterogeneous systems are expected provided the speed is sufficiently low for the heaviest component. Additional studies are needed to demonstrate whether further limitations over those already set forth here for homogeneous systems must be imposed when this procedure is applied to heterogeneous systems.

Acknowledgment

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Chemical and Biological Properties of Reduced and Alkylated Staphylococcal Enterotoxin B*

John E. Dalidowicz, Sidney J. Silverman, Edward J. Schantz, David Stefanye, and Leonard Spero

ABSTRACT: The single disulfide bridge in staphylococcal enterotoxin B has been found to be nonessential for the biological activity and conformation of the protein. Reduction of the disulfide bridge and alkylation of the resulting SH groups with both iodoacetamide and iodoacetate produced derivatives that had the same emetic activity and immunological properties as the native enterotoxin. Reduction and reoxidation of the disulfide bridge again did not produce any immunological or emetic changes. The physical properties of the alkylated enterotoxins and the reduced and oxidized enterotoxin, as measured by viscosity and sedimentation, remained essentially the same as that of the native enterotoxin. Unfolding in 6 M guanidine hydrochloride gave equivalent viscosities for the native and reduced carboxamidomethylated enterotoxins, and the derivative, after removal of the guanidine, regained full biological activity.

Staphylococcal enterotoxins are the substances elaborated by *Staphylococcus aureus* and responsible for emesis and diarrhea in food poisoning. Staphylococcal enterotoxin B has been prepared in a highly purified state by Schantz *et al.* (1965). It has a molecular weight of 35,300 and contains 1.04% sulfur, equivalent to 12 sulfur-containing amino acid residues per molecule. No free sulfhydryl was found either as *S*-carboxymethylcysteine or with titrations using *p*-mercuribenzoate. All the sulfur in the molecule is accounted for by ten methionine residues and two half-cystine residues forming one disulfide bridge per molecule (Spero *et al.*, 1965).

The present communication is concerned with the reduction of the disulfide bridge and the alkylation of the resulting sulfhydryl groups with iodoacetamide and iodoacetate. It also includes studies on the reoxidation of the sulfhydryl groups of reduced enterotoxin. The physical properties and immunological and emetic

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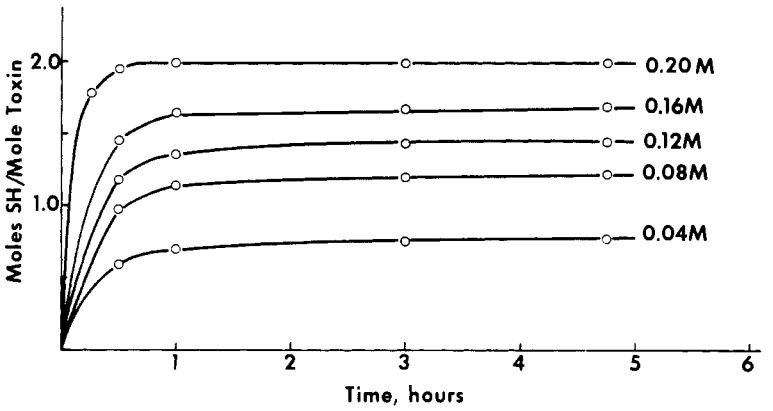

FIGURE 1: Reduction of staphylococcal enterotoxin B by β-mercaptoethanol. The reduction was carried out at room temperature under nitrogen using 25 mg of enterotoxin dissolved in 3.0 ml of 1.4 M Tris buffer, pH 8.6, containing 2 mg/ml of EDTA. The indicated molarity is that of β-mercaptoethanol.

activities of the *S*-carboxymethyl,[1] *S*-carboxamidomethyl, and the reduced–oxidized enterotoxins are compared with native staphylococcal enterotoxin B.

Experimental Procedure

Materials. Staphylococcal enterotoxin B was prepared by the method of Schantz *et al.* (1965). β-Mercaptoethanol was obtained from Mann Research Laboratories and was redistilled before use. Iodoacetamide and iodoacetate were obtained from Calbiochem and were recrystallized before use. 5,5′-Dithiobis-(2-nitrobenzoic acid) was obtained from Aldrich Chemical Co. and used without further purification.

Methods. REDUCTION AND ALKYLATION OF STAPHYLOCOCCAL ENTEROTOXIN B. A modification of the method of Crestfield *et al.* (1963) was used for the reduction of the disulfide bridge in staphylococcal enterotoxin B. The enterotoxin (25–100 mg) was dissolved in 3 ml of Tris buffer, pH 8.6 (5.23 g of Tris and 9 ml of 1.0 N HCl diluted to 30 ml with water), containing 2 mg/ml of EDTA. To this, 0.12 ml of redistilled β-mercaptoethanol was added and the vessel was flushed with nitrogen and stoppered. Urea was not required in the reduction. After 1 hr the reaction mixture was desalted on a calibrated 1 × 15 cm column of Sephadex G-25. The test tube in which the reduced toxin was collected was continuously flushed with nitrogen to minimize the reoxidation of the exposed sulfhydryl groups.

The desalted, reduced toxin was then adjusted to pH 7.3 and 10–50 mg of iodoacetamide or iodoacetate was added. The iodoacetamide was added in solid form; the iodoacetate was added in 0.5 ml of H_2O adjusted to pH 7.3. The alkylation was then followed in a pH-Stat (Radiometer TTT1, Copenhagen) by the uptake of 2×10^{-3} M NaOH.

VISCOMETRY. Viscosity measurements were made in a Cannon–Ubbelohde semimicrodilution viscosimeter (Cannon Instrument Co., State College, Pa.) at 20°. The protein was dissolved either in 0.05 M phosphate buffer, pH 6.55, or in 6 M guanidine chloride buffered with 0.1 M phosphate, pH 6.4. All samples were dialyzed *vs.* the solvent before measurement and filtered through a 0.25-μ Millipore filter. Efflux times were over 200 sec for all measurements. Plots of $(\ln t/t_0)/c$ *vs.* c were extrapolated to zero concentration and corrected for density by the method of Tanford (1955) to obtain the limiting viscosity number. Partial specific volumes for the toxin and the derivatives in aqueous and guanidine solutions were taken as 0.743, the experimental value determined by Wagman *et al.* (1965).

BIOLOGICAL ASSAYS. Quantitative precipitin tests were performed according to the procedure of Heidelberger and Kendall (1929). Test material was incubated at 37° for 4 hr and then at 4° for 24 hr. Washed precipitates were dissolved in 0.25 N acetic acid and the total N was determined by ultraviolet spectrophotometry at 277 mμ. The amount of enterotoxin present was also determined by immunodiffusion in agar, using the technique of Oudin (1946) as described by Silverman (1963). Antitoxin was prepared by the extensive immunization of rabbits with enterotoxin emulsified in Freund's complete adjuvant. Tests for impurities were carried out by the Ouchterlony (1949) technique. Although Oudin and Ouchterlony tests do not measure toxicity *per se*, they correlate well with toxicity tests in monkeys for the native toxin. Toxicity of the preparations was assayed by intravenous injections into rhesus monkeys (*Macaca mulatta*, weight about 3 kg) to obtain the biological activity characterized by emesis and diarrhea. In conducting this research the animals were maintained in compliance with the principles established by the National Society for Medical Research.[2]

[1] Abbreviations used in this work: RCM, reduced carboxymethyl; RCAM, reduced carboxamidomethyl; RO, reduced and reoxidized; ED_{50}, effective dose for 50% of the animals.

[2] National Society for Medical Research Principles of Laboratory Animal Care.

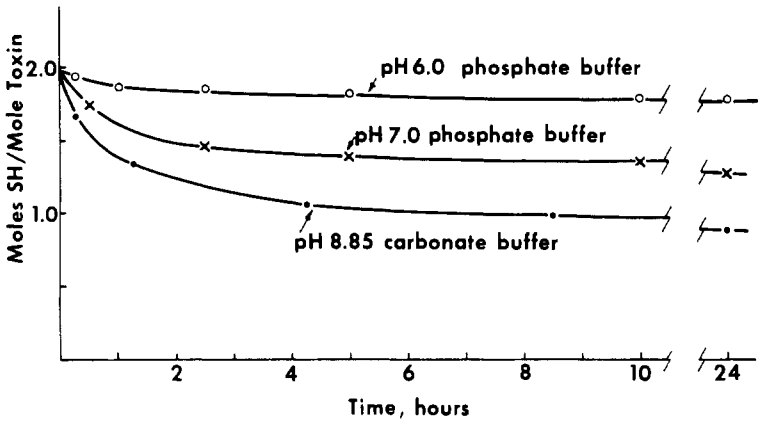

FIGURE 2: Oxidation of reduced staphylococcal enterotoxin B. Reduction was carried out as described under Figure 1. The Tris buffer was exchanged for 0.05 M phosphate or carbonate buffer on Sephadex G-25. Each buffer contained 2 mg/ml of EDTA. The enterotoxin concentrations were approximately 2.6 mg/ml. All oxidations were carried out at room temperature.

OTHER METHODS. Reduction of the disulfide bridge of staphylococcal enterotoxin B by β-mercaptoethanol was followed by measuring the release of sulfhydryl groups by the spectrophotometric method of Ellman (1959) with 5,5′-dithiobis(2-nitrobenzoic acid) after desalting on Sephadex G-25.

Concentration of enterotoxin was measured spectrophotometrically at 277 mμ on a Beckman DU spectrophotometer, using $E^{1\%}_{1\,cm}$ 14 (Schantz *et al.*, 1965). Amino acid analysis was done on a Phoenix automatic amino acid analyzer (Model 5000) according to the methods of Spackman *et al.* (1958).

Sedimentation constants were determined on a Spinco Model E ultracentrifuge, equipped with a rotor temperature indicating and control unit. In most experiments a capillary type synthetic boundary cell was used.

Results

Reduction of the Disulfide Bridge of Staphylococcal Enterotoxin B. Staphylococcal enterotoxin B was reduced by β-mercaptoethanol at pH 8.6 under nitrogen atmosphere. Preliminary experiments showed that urea was not required for the reduction. The rate of the reduction was followed by measuring the release of sulfhydryl groups by the method of Ellman (1959). Figure 1 shows that for full reduction of the disulfide bridge, the concentration of mercaptoethanol must be 0.2 M and the minimum time for reduction must be 1 hr. At the lower concentrations of mercaptoethanol, the reduction is incomplete and stops at a certain level, depending on the concentration of β-mercaptoethanol, after approximately 1 hr. Increase of reduction time has no significant effect on the amount of toxin reduced by mercaptoethanol at concentrations below 0.2 M.

The free sulfhydryl groups in the enterotoxin can be oxidized quite readily by letting the reduced enterotoxin stand at room temperature in air. The oxidation rate is dependent upon the type and pH of the buffer used. In phosphate buffer, pH 6.35, the oxidation proceeds to completion in 2 hr, but at pH 8.0 the oxidation of the reduced enterotoxin is very slow. In carbonate buffer, pH 9.5, the oxidation is complete in slightly more than 3 hr. The pH–buffer effect of the oxidation of the reduced enterotoxin is in accord with observations that the rates of oxidation of a given thiol vary considerably in different buffer solutions even though they are at the same pH (Fruton and Clarke, 1934).

Another complication arises from the fact that oxidation of thiols is strongly catalyzed by minute amounts of copper and iron (Fruton and Clarke, 1934; Elvehjem, 1930). The rate of oxidation, however, can be kept extremely low if these ions are removed with a chelating agent (Sakuma, 1923; Harrison, 1924). Figure 2 shows that after 24 hr, the toxin is not completely reoxidized in either phosphate or carbonate buffer when 2 mg/ml of EDTA is added to the buffer. The results at pH 6 are the most striking, going from the greatest lability to almost complete stability.

Alkylation of the Reduced Enterotoxin. After reduction the sulfhydryl groups were alkylated by either iodoacetamide or iodoacetate. The alkylation proceeds at a constant rate and is complete in approximately 1 hr with iodoacetamide and 3 hr with iodoacetate. This rate difference was observed by Smythe (1936) and attributed to either an easier nucleophilic displacement of the I^- from iodoacetamide than iodoacetate or a coulombic repulsion between the mercaptide and iodoacetate ion (Cecil and McPhee, 1959). Occasionally nearly quantitative alkylation was achieved but in most experiments only 75% of the toxin was alkylated, the remainder being reoxidized during the process. The oxidized toxin can be removed from the alkylated products by CM-cellulose chromatography at 3° with 0.05 M phosphate buffer, pH 6.1. Both alkylated derivatives are retained by CM-cellulose. The RCAM-toxin is eluted quite readily after a stepwise change to 0.2 M phosphate buffer at pH 6.4. The RCM-toxin,

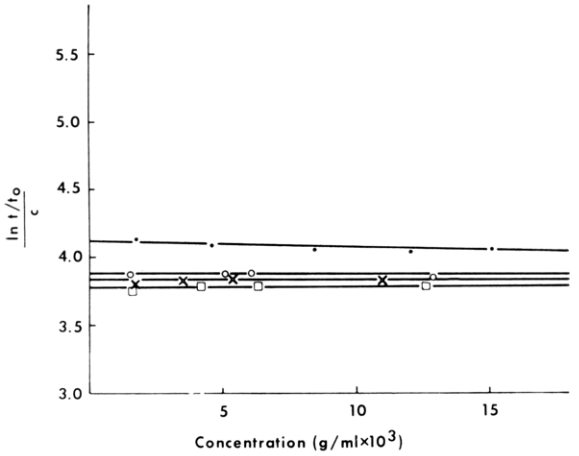

FIGURE 3: Viscosity of staphylococcal enterotoxin B and derivatives. The viscosities were determined at 20° in 0.05 M phosphate buffer, pH 6.55. ●, RCM-enterotoxin; ○, RCAM-enterotoxin; ×, RO-enterotoxin; and □, native enterotoxin.

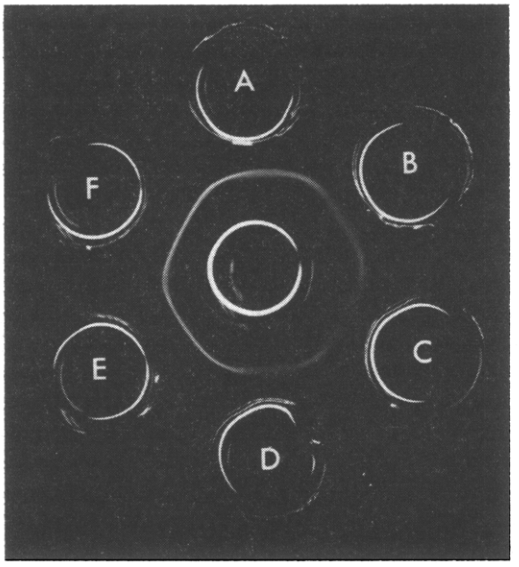

FIGURE 4: Comparison by Ouchterlony immunodiffusion of native enterotoxin, the three derivatives, and RCAM-enterotoxin after renaturation. The agar was made up in isotonic saline containing 0.5 M glycine. Center well contained a 1:5 dilution of antienterotoxin. All sample wells contained 50 μg/ml of protein. A and D, native enterotoxin; B, RCAM-enterotoxin which was denatured in 6 M guanidine and renatured by removal of the guanidine; C, RO-enterotoxin; E, RCAM-enterotoxin; and F, RCM-enterotoxin.

however, is not. Only a small amount of the derivative is obtained, the rest being retained on the column. This is true even if the pH of the buffer is increased to 7.3. The stronger binding to the resin suggests some degree of conformational change in the alkylated derivatives.

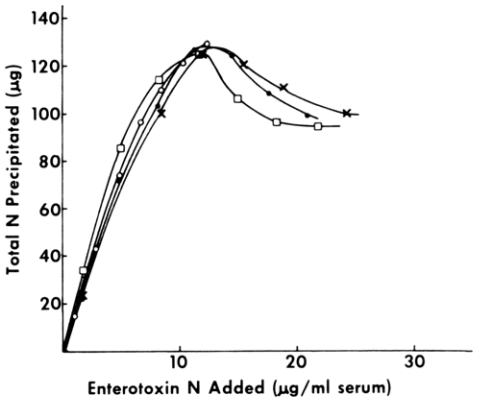

FIGURE 5: Precipitation of staphylococcal antienterotoxin B by altered and unaltered enterotoxin. The samples were incubated for 4 hr at 37° and then for 24 hr at 4°. □, native enterotoxin; ●, RCAM-enterotoxin; ×, RCM-enterotoxin; and ○, RO-enterotoxin.

The amino acid analysis of both the alkylated toxin derivatives after purification by CM-cellulose chromatography showed two *S*-carboxymethylcysteine residues per molecule of toxin. The amino acid analysis also showed no evidence that any histidine or lysine residues were alkylated in the process.

Physical Properties of the RCM- and RCAM-Enterotoxins. The physical properties of the two column-purified alkylated derivatives and the RO-toxin remained essentially the same as that of the unaltered enterotoxin. The rate of sedimentation in the analytical ultracentrifuge and the sedimentation pattern were essentially unchanged by the alkylation or reduction and oxidation. Table I shows that the sedimentation coefficients for the altered enterotoxins were between 2.6 and 2.8 S for concentrations of 5 mg/ml. This is in good agreement with the sedimentation coefficient obtained for an equivalent concentration of the native toxin (2.8 S) (Wagman *et al.*, 1965).

TABLE I: Physical Properties of Staphylococcal Enterotoxin B and Derivatives.

Sample	$s_{20,w}$ (S)	$[\eta]$ (ml/g)
Native enterotoxin	2.80	4.03
RCAM-enterotoxin	2.71	4.13
RCM-enterotoxin	2.68	4.37
RO-enterotoxin	2.60	4.09
Native enterotoxin in 6 M guanidine	..	25.9
RCAM-enterotoxin in 6 M guanidine	..	27.2
Native enterotoxin after removal of guanidine	..	4.08
RCAM-enterotoxin after removal of guanidine	..	4.19

TABLE II: Biological Activity of Staphylococcal Enterotoxin B and Derivatives.

	Serolog. Act., μg of Act. Toxin/100 μg of Protein		Rhesus Monkey Response				
			0.1 μg/kg		0.3 μg/kg		
	Precipitin Tests	Immunodiffusion Tests	Diarrhea and Emesis[a]	Latent Period (min)	Diarrhea and Emesis	Latent Period (min)	Estimated ED_{50} (μg/kg)
Untreated enterotoxin	121	101	~50%	66–152	5/6	60–120	0.1
RCAM-enterotoxin	121	117	1/3	111	2/3	65–100	<0.3
RCM-enterotoxin	117	122	1/3	175	4/6	82–89	<0.3
RO-enterotoxin	122	118	0/3	...	5/6	52–121	<0.3
Renatured RCAM-enterotoxin	...	...	1/4	179	3/4	83–108	<0.3

[a] Where response is given as a fraction, the numerator refers to the number of animals responding and the denominator refers to the total number of animals tested.

The conformational change in the alkylated derivatives, especially the RCM-enterotoxin, suggested by their altered behavior during CM-cellulose chromatography is also reflected in their viscosity. Figure 3 shows a plot of $(\ln t/t_0)/c$ *vs.* concentration for the native and altered toxins. The lines have zero slope, indicating that there is no association of the alkylated toxin molecules. Extrapolation of these plots to zero concentration and correction for density yielded the limiting viscosity numbers shown in Table I. The limiting viscosity number of the native toxin was 4.03. The RCAM-enterotoxin and the RO-enterotoxin gave values of 4.13 and 4.09, respectively. The RCM-enterotoxin gave a limiting viscosity number of 4.37. A calculation of the axial ratios for the molecules as anhydrous prolate ellipsoids shows an 8% difference for the RCM-enterotoxin but less than 3% for the other two toxins in comparison with the native molecule.

Electrophoresis of the RCAM-enterotoxin on starch gel using 0.02 M borate buffer at pH 8.6 showed one component. Native enterotoxin gives two major components in starch gel electrophoresis (Schantz *et al.*, 1965; Baird-Parker and Joseph, 1964). The mobility of the alkylated enterotoxin was found to be slower than that of any of the components of the native enterotoxin.[3]

Biological Properties of the Alkylated Enterotoxins. The alkylated derivatives and the RO-enterotoxin were compared with the native enterotoxin for biological activity by precipitin, immunodiffusion, and emetic tests. Figure 4 shows that a reaction of identity was obtained with native, RCM-, RCAM-, and RO-enterotoxins in Ouchterlony double diffusion. Figure 5 shows that the precipitin curves for the alkylated and the RO-enterotoxins were basically the same as that of the unaltered enterotoxin. The Oudin immunodiffusion tests (Table II) also show that the altered enterotoxins have the same serological activity as the native enterotoxin. The movement of the enterotoxins in the immunodiffusion tests was the same for all derivatives tested. Emetic tests on rhesus monkeys indicated that the two alkylated derivatives and the RO-enterotoxin have ap-

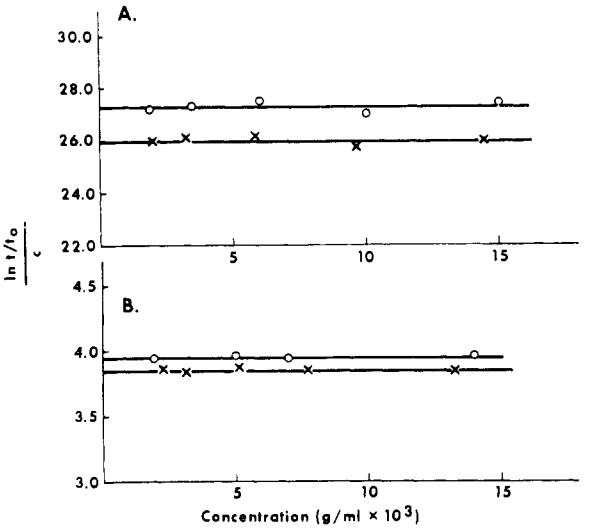

FIGURE 6: Viscosity of denatured and renatured staphylococcal enterotoxin B and RCAM-enterotoxin. A, proteins in 6 M guanidine hydrochloride; B, proteins in 0.05 M phosphate buffer, pH 6.55, after removal of guanidine by gel filtration on Sephadex G-25. O, RCAM-enterotoxin; ×, native enterotoxin.

[3] Since the RCAM-enterotoxin used in this experiment was obtained quantitatively from the toxin, these results suggest very strongly that the separations found with the native enterotoxin by Schantz *et al.* (1965) and Baird-Parker and Joseph (1964) do not reflect components with differing primary structure. Differences in the number of amide groups or amino acid substitutions would persist in the alkylated derivatives.

proximately the same effective dose for 50% of the animals (ED_{50}) for emesis as that of the native enterotoxin. Table II shows that of 15 monkeys tested only four failed to develop either vomiting or diarrhea at a dose of 0.3 μg/kg. The latent period of reaction for the altered enterotoxins was of the same range as that of the regular toxin. At a dose of 0.1 μg/kg one of three monkeys for both RCM- and RCAM-enterotoxins gave a positive response. The RO-enterotoxin at this level gave negative results. A statistical evaluation of the ED_{50} for emesis in monkeys for the native enterotoxin gave a value of 0.1 μg/kg with 95% confidence limits of 0.05–0.2 (Schantz *et al.*, 1965). The limited number of animals involved in the testing of the altered toxins precludes an accurate evaluation of their ED_{50}, but the data, emetic response, and latent period strongly suggest equivalent activities.

Reversible Denaturation of RCAM-Enterotoxin. The RCAM- and native enterotoxin were dissolved in 6 M guanidine hydrochloride buffered with 0.1 M phosphate buffer, pH 6.4. After standing for 4 hr at room temperature, the viscosity was measured. Figure 6 shows a plot of $(\ln t/t_0)/c$ *vs.* c. A limiting viscosity number of 27.2 was obtained for the RCAM-enterotoxin. Similarly, native toxin in 6 M guanidine hydrochloride gave a limiting viscosity number of 25.9. The guanidine was then removed by dialysis or by gel filtration, the solution was lyophilized, the residue was dissolved in 0.05 M phosphate buffer pH 6.55, and the viscosity was again determined. Limiting viscosity numbers of 4.19 and 4.08 for the RCAM-enterotoxin and native enterotoxin were obtained. These correspond very closely to the limiting viscosity numbers of the derivative (4.13) and native toxin (4.03) that were obtained without any guanidine treatment.

Ouchterlony tests (Figure 4) on the guanidine-treated and subsequently dialyzed RCAM-enterotoxin showed reactions of identity with undenatured RCAM- and native enterotoxins as controls. The renatured RCAM-toxin was tested for biological activity by intravenous injection in rhesus monkeys. Table II shows that, at a dose of 0.3 μg/kg, three of four monkeys developed emesis. When the dose was decreased to 0.1 μg/kg, one monkey of four developed emesis. The latent period of reaction was of the same range as that of the native enterotoxin. The renatured RCAM-enterotoxin is, therefore, biologically and serologically equivalent to the untreated derivative.

Discussion

The data show that staphylococcal enterotoxin B may be reduced and reoxidized without producing an important change in conformation or biological activity. Furthermore, reduction and alkylation yielded derivatives with equivalent emetic and serological activity and only slightly altered physical properties. Some degree of conformational change in the alkylated derivatives is suggested, though, by their altered behavior during CM-cellulose chromatography. Both derivatives are bound to the carboxylic acid resin more strongly than the native material despite the fact that introduction of the carboxamidomethyl groups does not alter the number of charged groups on the molecule and that the introduction of the carboxymethyl groups increases the number of anionic groups by two. It is apparent that several cationic or hydrogen-bonding groups are made available for interaction with the resin as a result of the reduction and alkylation process. The conformation of the RCM-enterotoxin is more strongly affected, presumably because of the repulsive effect of the two carboxyl functions. This derivative is held much more strongly to the resin and has a higher limiting viscosity number. Although these slight changes are real they do not indicate a drastic conformational alteration. Furthermore, these alterations must be confined to small areas of the protein surface; the sites involved in both emetic activity and combination with specific antibody remain unchanged.

Viscosity measurements show that the disulfide bridge of staphylococcal enterotoxin B is not necessary for the conformational structure of the molecule. Reduction and alkylation produce only minor changes in axial ratio. In addition, the disulfide loop provides very little structural restraint to the molecule, possibly indicating that the two half-cystine residues are not widely separated in the polypeptide chain. Unfolding in 6 M guanidine gave essentially equivalent limiting viscosity numbers for the native and RCAM-enterotoxin. This is in marked contrast to other proteins such as ribonuclease (Harrington and Sela, 1959) and serum albumin (Frensdorff *et al.*, 1953) where the disulfide bridges provide significant configurational restraint in the presence of denaturants. The disulfide bridge is also not necessary for the biological activity of the enterotoxin because the alkylated enterotoxins have equivalent emetic and serological activity. Furthermore, the refolding of the RCAM-enterotoxin upon removal of the denaturing agent after treatment with 6 M guanidine is probably the same as that of the native toxin in that the renatured RCAM-enterotoxin has the same serological activity, measured by Ouchterlony tests, and the same biological activity, measured by emesis in rhesus monkeys, as the native enterotoxin. Thus, in common with a growing list of proteins (Epstein *et al.*, 1963), all of the information for the formation of biologically active enterotoxin resides in the amino acid sequence, and the introduction of carboxamidomethyl groups on the sulfhydryls does not interfere with its refolding.

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Formation of Methane from Methyl Factor B and Methyl Factor III by Cell-Free Extracts of *Methanobacillus omelianskii**

J. M. Wood, M. J. Wolin, and R. S Wolfe

ABSTRACT: Methylcobinamide (methyl factor B) and methyl-Co-5-hydroxybenzimidazolylcobamide (methyl factor III) were tested as methyl donors for the transmethylation reaction leading to the formation of methane in extracts of the methane bacterium *Methanobacillus omelianskii*. The methyl-cobalt ligands of both substrates were approximately as active as that of methyl-Co-5,6-dimethylbenzimidazolylcobamide (methylcobalamin); the presence of the dimethylbenzimidazole nucleotide moiety is not required for the methyl–cobalt ligand to be active as methyl donor Adenosine triphosphate was required for the formation of methane from both methyl-B_{12} analogs. The properties of derivatives prepared from the respective reduced cobinamide and cobamide products which accumulate suggest that they possess sulfhydryl radicals on their respective cobalt atoms. The striking lack of specificity of methyl-B_{12} analogs as methyl donors in biological systems is postulated to reside in their ability to chemically alkylate reduced corrinoid-containing enzymes.

To test the specificity of the enzyme system in *Methanobacillus omelianskii* which converts methylcobalamin[1] to methane (Wolin *et al.*, 1963, 1964), methyl factor B (Müller and Müller, 1962) and methyl factor III were chosen as substrates. Methyl factor B may be regarded as methylcobalamin with the exclusion of the benzimidazole nucleotide moiety, and factor III derivatives have been shown to be the predominant, naturally occurring cobamide compounds present in *M. omelianskii* (Lezius and Barker, 1965).

Experimental Section

Factors B and III were generous gifts from Dr. L. Mervyn of Glaxo Research Limited, Greenford, Middlesex, England. Methyl factor B was synthesized by methylating hydrido factor B with dimethyl sulfate at 0°, and its absorption spectrum was compared with a sample of methyl factor B kindly provided by Dr. D. H. Dolphin. Spectra were determined by use of a Cary Model 14 spectrophotometer. Hydrido factor B was

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[1] Abbreviations used in this work: methyl factor B, methylcobinamide; methyl factor III, methyl-Co-5-hydroxybenzimidazolylcobamide; methylcobalamin, methyl-Co-5,6-dimethylbenzimidazolylcobamide; ATP, adenosine 5′-triphosphate.