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Unique chemical reactivity of His-21 of CRM-197, a mutated diphtheria toxin

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Abstract CRM-197 is a mutated diphtheria toxin (63 000 Da) widely used as a carrier protein of conjugated vaccines. Among the 14 histidines of CRM-197, His-21 was found to be modified selectively with iodoacetamide based reagents. This finding suggests a simplified method for the preparation of conjugate vaccines crosslinked to CRM-197. A bifunctional iodoacetamide, *N*,*N*′-(2-hydroxy-1,3-propanediyl)-bis-[2-iodoacetamide] (I-CH_2-CONH-CH_2-CH(OH)-CH_2-NHCO-CH_2-I) (HPBIA), was synthesized and allowed to react with CRM-197. In the alkaline buffer of pH 8.0–8.4, HPBIA was shown to react and intra-bridge His-21 and Lys-24 of CRM-197 sequentially. At lower pH (7.1–7.5) in the phosphate buffer, the reactivity of Lys-24 toward HPBIA was suppressed drastically. Under these conditions, His-21 could be specifically labeled with HPBIA. Initial experiments have demonstrated that HPBIA modified CRM-197 is able to crosslink to a cysteine-containing peptide. These results offer a potential route for improving the homogeneity of CRM-197 based protein-peptide as well as protein-polysaccharide conjugates.

Key words: Reactive histidine; CRM-197; Chemical modification; 4-*N*,*N*-dimethylaminoazobenzene-4′-iodoacetamido-2′-sulfonic acid; *N*,*N*′-(2-hydroxy-1,3-propanediyl)-bis-[2-iodoacetamide]

1. Introduction

CRM-197 is a mutated, non-toxic diphtheria toxin [1,2]. A single G→A mutation which leads to the substitution of Gly-52 by Glu distinguishes CRM-197 from its wild-type species. The absence of toxicity of CRM-197 [3,4] is due to the loss of enzymatic activity of its fragment A, which in the wild type catalyzes the chemical modification of elongation factor 2 (translocase) in infected cells that is essential for protein synthesis. This unique non-toxic property, together with its potent immunogenicity, has made CRM-197 a popular carrier protein for preparation of conjugated vaccines [5–10].

Conjugation with CRM-197 has been typically achieved through activation of the lysyl residues using various types of bifunctional crosslinkers. Since CRM-197 contains 40 lysines and many of them are available for crosslinking, the end products of CRM-197 conjugates are invariably heterogeneous. For instance, one of such conjugated vaccines is a novel anti-idiotype antibody vaccine for melanoma (CGP-62360) prepared in Novartis [11]. CGP-62360 was produced by crosslinking CRM-197 via lysine residues to the cysteines of a partially reduced antibody (IgG2b). Among the 40 total lysines of CRM-197, at least 5 have been shown to be highly reactive and could be preferentially labeled with a lysine specific reagent, 4-*N*,*N*-dimethylaminoazobenzene-4′-isothiocyano-2′-sulfonic acid (S-DABITC) [12]. On the antibody site, a minimum of two disulfide bonds become partially reduced even under mild reducing conditions (1–5 mM of dithiothreitol, 15 min at room temperature) (Knecht and Chang, unpublished data). The multiple crosslinking sites of both CRM-197 and the antibody account for the complexity of the conjugate formed. CGP-62360 indeed consists of highly heterogeneous molecular species. This has been characterized by capillary electrophoresis, mass spectrometry, high resolution electron microscope, size-exclusion and reversed phase chromatography (O'Reilly et al., manuscript in preparation). Even in the cases of conjugation with small molecular weight moieties, the multiple activation sites of CRM-197 alone are expected to generate a high degree of heterogeneity in the end product.

As a consequence of attempts to improve the homogeneity of the CRM-197 conjugate, we have come across the finding that histidine residue 21 of the CRM-197 could be selectively modified by reagents that possess functional iodoacetamido groups. In addition, we have also observed that His-21 and Lys-24 of CRM-197 can be intra-crosslinked and bridged by a bifunctional iodoacetamide. These properties signal the unique chemical environment surrounding His-21 and suggest a potentially useful route of preparing CRM-197 conjugates with greatly reduced heterogeneity. In this report, we will describe conditions for the selective modification of His-21 and the synthesis and application of a crosslinker that was used to modify His-21 and bridge His-21 and Lys-24 of CRM-197.

2. Experimental procedures

2.1. Materials

CRM-197 is a mutated wild-type diphtheria toxin. The protein was supplied by Chiron/Biocine (Siena, Italy) and has a purity of 99.3% judged by HPLC. A wild-type diphtheria toxin was purchased from Sigma. The S-DABIA was synthesized and prepared according to the method described previously in our laboratory [13]. A cysteine containing peptide (Lys-Asn-Gln-Cys-Val-Thr-Gly-Glu) corresponding to the hirudin sequence residues 36–43 was synthesized by solid phase method and was kindly supplied by Dr. Hans Rink. TPCK treated trypsin was purchased from Sigma. All other chemicals used for the preparation of buffers were obtained from Fluka with a minimum purity of higher than 99%.

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Abbreviations: CRM 197, mutated non-toxic diphtheria toxin; HPLC, high performance liquid chromatography; MALDI, matrix-assisted laser desorption ionization; PTH, phenylthiohydantoin; HPBIA, *N*,*N*′-(2-hydroxy-1,3-propanediyl)-bis-[2-iodoacetamide]; TFA, trifluoroacetic acid; S-DABIA, 4-*N*,*N*-dimethylaminoazobenzene-4′-iodoacetamido-2′-sulfonic acid

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2.2. Reaction of CRM-197 with S-DABIA

CRM-197 (0.2 mg) was dissolved in 0.2 ml of alkaline buffer containing 0.3 mg of S-DABIA. The alkaline buffer was either 0.1 M sodium bicarbonate solution (pH 8.3) or 25 mM sodium phosphate solution (pH 8.2). Derivatization was carried out at 23°C in a time course manner. The samples were then passed through a NAP-5 column (Pharmacia) and eluted with ammonium bicarbonate buffer (50 mM, pH 7.9). The eluted samples were directly subjected to trypsin digestion.

2.3. Trypsin digestion of S-DABIA labeled CRM-197

S-DABIA derivatized CRM-197 was digested with trypsin in the ammonium hydrogen carbonate solution (50 mM, pH 7.9) at 23°C for 24 h. The final protein concentration was 0.3 mg/ml. The ratio of the enzyme/substrate by weight was 1:10. After digestion, the sample was acidified with 4% trifluoroacetic acid and was ready for HPLC injection.

2.4. HPLC analysis of S-DABIA labeled peptides

The chromatographic equipment was a Hewlett Packard 1090 Liquid Chromatograph with diode array detector. The mobile phases used were: Phase A, 0.1% trifluoroacetic acid (TFA) purchased from Pierce, in millipore water. Phase B was 90% gradient grade acetonitrile, 10% millipore water and 0.08% TFA. The gradient was 15% B to 60% B in 70 min. The flow rate was 200 μl per min. The column was Vydac C18, 5 μm, 25 cm, for peptides and proteins. S-DABIA derivatized peptides were detected at 536 nm, isolated from HPLC and characterized by amino acid sequencing and MALDI mass spectrometry.

2.5. Synthesis of N,N′-(2-hydroxy-1,3-propanediyl)-bis-[2-iodoacetamide], I-CH_2-CONH-CH_2-CH(OH)-CH_2-NHCO-CH_2-I (HPBIA)

1,3-Diaminoisopropanol (0.47 g, 5 mM) was dissolved in 15 ml of acetonitrile/water (1:1, by volume). 4-Nitrophenyliodoacetate (3.08 g, 10 mM) was added in portions by constant stirring. The reaction temperature was kept below 24°C using a water bath. After 80 min of reaction, acetonitrile (10 ml) was added to precipitate the yellowish mixture. The precipitate was filtered, washed twice with 10 ml of acetonitrile and finally dried in vacuo for 24 h. The yield was 575 mg (27%). Molecular mass found by MALDI-TOF 426.5 (expected: 426). Elementary analysis: Found (expected): C: 19.79% (19.74%); H: 3.12% (2.84%), N: 6.60% (6.58%); I, 59.27% (59.58%).

2.6. Reaction of CRM-197 with HPBIA

CRM-197 (0.2 mg) was dissolved in 0.2 ml of the buffer solution containing 0.2 mg of HPBIA. The buffer was either 0.1 M sodium bicarbonate solution (pH 8.3) or 25 mM sodium phosphate solutions (pH 8.3, 7.9, 7.5 and 7.1, respectively). Derivatization was carried out at 23°C in a time course manner for each buffer solution. After the reaction, the samples were passed through a NAP-5 column (Pharmacia), eluted with the sodium bicarbonate buffer (50 mM, pH 8.3), collected in a volume of 550 μl and immediately introduced with 1 μl of β-mercaptoethanol in order to block the unreacted iodoacetamide of HPBIA. The samples were again passed through a NAP-5 column (Pharmacia), eluted with ammonium bicarbonate buffer (50 mM, pH 7.9) and directly subjected to the trypsin digestion.

2.7. Structural characterization of HPBIA labeled CRM-197

Conditions for trypsin digestion and HPLC analysis of HPBIA derivatized CRM-197 were similar to those described for the analysis of S-DABIA labeled samples.

2.8. Crosslinking of HPBIA activated CRM-197 to a cysteine containing peptide

CRM-197 (0.2 mg) was allowed to react with 0.2 mg of HPBIA in 0.2 ml of sodium phosphate buffer (25 mM, pH 7.1). Derivatization was carried out at 23°C for 3 h. After the reaction, the samples were passed through a NAP-5 column (Pharmacia), equilibrated and eluted with the phosphate buffer (50 mM, pH 7.5). HPBIA activated CRM-197 was collected in a volume of 550 μl in an Eppendorf tube containing 0.2 mg of the lyophilized octapeptide (Lys-Asn-Gln-Cys-Val-Thr-Gly-Glu). After 1 h of reaction, the sample was again passed through a NAP-5 column (Pharmacia), eluted with ammonium bicarbonate buffer (50 mM, pH 7.9) and directly subjected to sequence analysis.

2.9. Amino acid sequence analysis and MALDI mass spectrometry

Amino acid sequences of isolated tryptic peptides were determined by a Hewlett Packard G 1000A sequencer equipped with an on-line PTH analyzer. All chemicals used to operate the sequencer were also obtained from Hewlett Packard. An internal standard, 2-nitroacetophenone, which is eluted in between PTH-Ala and PTH-Tyr, was introduced to ensure precise quantitation of PTH-amino acid. The MALDI mass spectrometer was a home-built time of flight (TOF) instrument with a nitrogen laser of 337 nm wavelength and 3 ns pulse width. The calibration was performed either externally or internally, by using standard polypeptides (hypertensin, MW 1031.19; synacthen, 2934.50 and calcitonin, 3418.91).

3. Results

3.1. His-21 of CRM-197 can be selectively modified with S-DABIA

The time course of CRM-197 reaction with S-DABITC was examined. At the desired times, the derivatized samples were removed from the excess reagent. The extent of S-DABIA labeling (mole of S-DABIA per mole of CRM-197) can be estimated from the absorbance of the samples at 465 nm based on the molar extinction coefficient of S-DABIA (29 000 at 465 nm and pH 8.0) [13]. Derivatized samples were subsequently digested with trypsin. Tryptic peptides were then analyzed by HPLC and monitored simultaneously at 214 nm and 536 nm in order to detect the S-DABIA labeled peptides. In both sodium bicarbonate and sodium phosphate buffers, only one major colored peptide was detected (Fig. 1). Recovery of this colored peptide reaches a plateau after 2–3 h of reaction. Sequence analysis and mass analysis unambiguously demonstrate that this peptide corresponds to residues 11–33 of CRM-197, with His-21 labeled with S-DABIA.

It is relevant to mention that the unique reactivity of His-21 is dependent upon the conformation of CRM-197. In the presence of denaturant (5 M GdmCl), or in case that the protein is fully reduced and carboxymethylated, the reactivity of His-21 is no longer detectable with S-DABIA (data not shown).

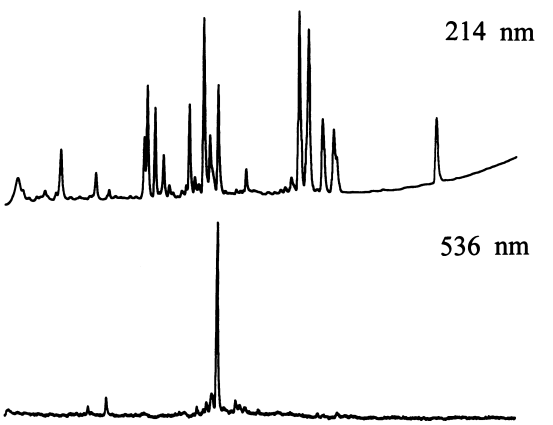

Fig. 1. HPLC tryptic peptides mapping of mutated diphtheria toxin (CRM-197) derivatized with S-DABIA. S-DABIA derivatization was performed in the sodium bicarbonate buffer (0.1 M, pH 8.3) at 23°C for 90 min. Additional reaction conditions and HPLC conditions are described in the text. Peptides were monitored at both 214 nm and 536 nm. Only one major color peptide eluted at 29.6 min was recovered. Structural analysis revealed that this peptide corresponded to residues 11–33 of CRM-197 with His-21 selectively modified with S-DABIA.

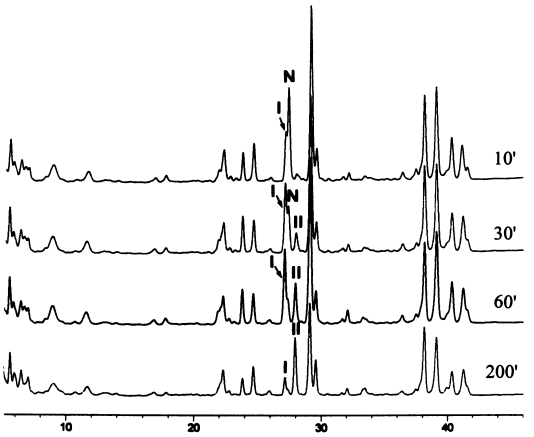

Fig. 2. HPLC tryptic peptides mapping of CRM-197 derivatized with HPBIA in a time course manner. HPBIA derivatization was performed in the sodium bicarbonate buffer (0.1 M, pH 8.3) at 23°C. Conditions for enzyme digestion and HPLC separation are described in the text. Peptides were monitored at 214 nm. Recovery of three major peptide fractions, marked **N**, **I** and **II** varies along the course of reaction. Their structures are shown in Fig. 3.

3.2. Synthesis of iodoacetamide based homo-crosslinkers

The finding that His-21 of CRM-197 can be selectively modified by iodoacetamide suggests that as a carrier protein CRM-197 may be crosslinked to a peptide or protein vaccine via His-21 alone, thus greatly reducing the heterogeneity of the CRM-197 conjugate. A homo-crosslinker possessing bifunctional iodoacetamide was required for this study. We initially synthesized a *N*,*N*′-bis-iodoacetamide with a trimethylene spacer. This reagent, however, proved to be unsatisfactory due to its limited solubility in the aqueous solution at alkaline and neutral pH. Therefore a hydroxyl group was introduced and a water soluble crosslinker, *N*,*N*′-(2-hydroxy-1,3-propanediyl)-bis-[2-iodoacetamide] (HPBIA) was prepared. The structure and purity of HPBIA was confirmed by HPLC, NMR, IR, molecular mass and elementary analysis. The purity of HPBIA is higher than 98%.

3.3. Intra-molecular bridging of His-21 and Lys-24, and selective modification of His-21 by HPBIA

Reaction of HPBIA with CRM-197 was first performed in the sodium bicarbonate buffer (0.1 M, pH 8.3). At different time intervals, the reaction was terminated by passing aliquots of the sample through gel filtration and excess iodoacetamide was blocked with excess β-mercaptoethanol. The samples were then digested with trypsin and analyzed by HPLC. Analysis of time-course trapped samples (Fig. 2) reveals a progressive decrease of fraction **N**, a transient intermediate denoted **I** that ascends and then descends along the course of reaction and the subsequent build-up of fraction **II**. These three fractions were characterized by both amino acid sequencing and molecular mass analysis. Sequence analysis by Edman chemistry confirms that fraction **N** contains a single peptide corresponding to residues 11–33 of CRM-197. His-21 and Lys-24 were recovered quantitatively as derivatives of phenylthiohydantoin at degradation cycles of 11 and 14. Fractions **I** and **II** reveal identical amino acid sequences. However, His is absent from cycle 11 in the case of fraction **I**. With fraction **II**, gaps were observed at both cycles of 11 and 14. Using MALDI mass spectrometry, **N**, **I** and **II** display molecular masses of

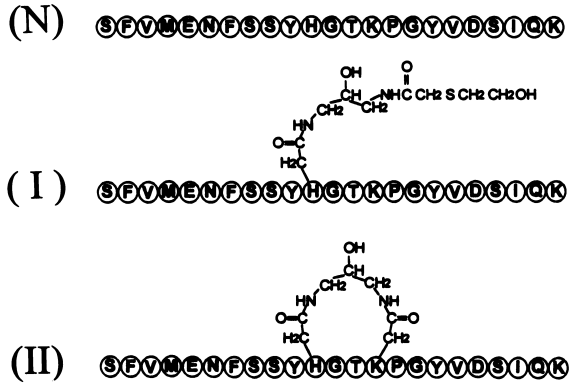

Fig. 3. Structures HPBIA modified peptides of CRM-197. (**N**) is the native sequence residues 11–33 of CRM-197. (**I**) is the same peptide with His-21 selectively modified with HPBIA (the other end of HPBIA is coupled to β-mercaptoethanol). (**II**) represents the same peptide with His-21 and Lys-24 crosslinked by HPBIA. The structures were confirmed by both amino acid sequencing and mass analysis.

2622, 2872 and 2793, respectively. These data, taken together, are consistent with the structures drawn in Fig. 3. **N** is the native peptide encompassing residues 11–33 of CRM-197. **I** represents the same peptide with single modification of His-21 by HPBIA and **II** corresponds to the same peptide with His-21 and Lys-24 intra-crosslinked by HPBIA. These results thus vividly demonstrate a sequential modification and bridging of His-21 and Lys-24 by HPBIA.

For the purpose of protein conjugation, however, the reaction must end at the single modification of His-21 and the formation of fraction **I** alone. Rationally, this may be achieved by optimizing the reaction conditions. Therefore, a systematic study by employing different buffers of varied pH was performed. The replacement of sodium bicarbonate buffer by phosphate buffer was shown to slow down the modifica-

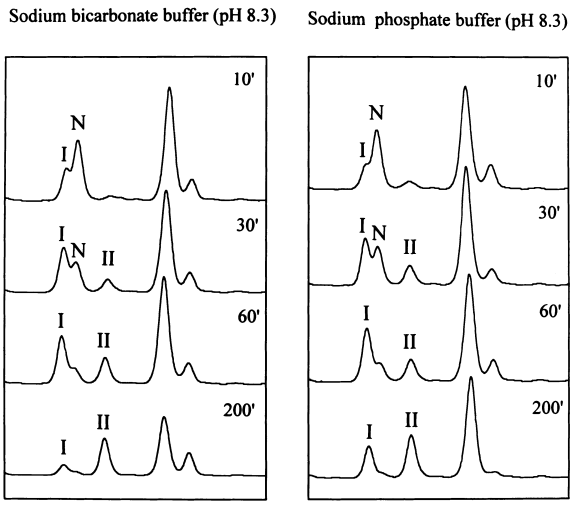

Fig. 4. HPLC tryptic peptides mapping of CRM-197 derivatized with HPBIA in the sodium bicarbonate buffer (left column) and sodium phosphate buffer (right column). HPBIA derivatization was carried out in a time course manner at 23°C. The concentration of buffer is 1 M and pH is 8.3. Conditions for enzyme digestion and HPLC separation are described in the text. Peptides were monitored at 214 nm. Only the window of the HPLC chromatogram between 26 and 32 min is shown here. Recoveries of three major peptide fractions (**N**, **I** and **II**) along the course of reaction are indicated.

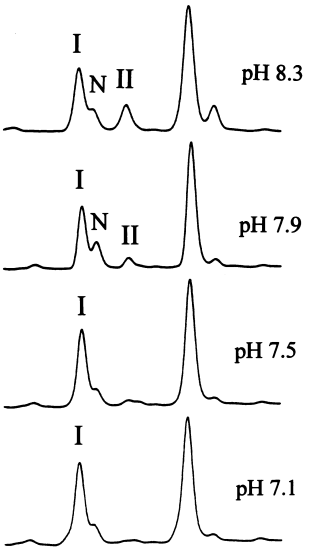

Fig. 5. Effect of pH on the selective modification of His-21 and Lys-24 of CRM-197 by HPBIA. HPBIA derivatization was carried out in the phosphate buffer (0.1 M) at 23°C for 60 min. Conditions for enzyme digestion and HPLC separation are described in the text. Peptides were monitored at 214 nm. Again, only the window of HPLC chromatogram between 26 and 32 min is shown here. Recoveries of three major peptide fractions (**N**, **I** and **II**) along the course of reaction are indicated.

tion of Lys-24. At the same pH (8.3) and otherwise identical reaction conditions (3 h, 23°C), the application of phosphate buffer reduced the rate of conversion of fraction **I** to **II** by a factor of 2 (Fig. 4). Lowering the pH of phosphate buffer has an even more profound effect of hindering the reaction with Lys-24. At pH in-between 7.1–7.5, reaction of CRM-197 with HPBIA occurs almost exclusively at His-21 (Fig. 5).

Conditions (phosphate buffer, pH 7.1) that permit selective modification of His-21 by HPBIA was applied to prepare HPBIA activated CRM-197, which was subsequently allowed to react with a model cysteine-containing peptide (Lys-Asn-Gln-Cys-Val-Thr-Gly-Glu). After removal of the excess reagent, the peptide conjugated CRM-197 was analysed by mass spectrometry and Edman degradation. The results reproducibly demonstrated a coupling yield of 25–30% of the cysteine peptide to the CRM-197.

4. Discussion

The X-ray structure of diphtheria toxin has revealed that His-21 and Lys-24 are situated within the enzymatic cleft of the catalytic domain [14]. The region surrounding His-21 and Lys-24 also bears sequence homology with other toxins that function as ADP-ribosyltransferase [15,16]. Furthermore, it has been demonstrated that His-21 of diphtheria toxin can be selectively modified with diethyl pyrocarbonate (a histidine specific reagent) and that this modification abolishes the enzymatic activity of diphtheria toxin [17]. Further study using mutagenesis has shown that His-21 is important in maintaining the steric conformation required for catalytic activity of the toxin [18]. CRM-197 is a diphtheria toxin related protein with only single amino acid substitution. Despite the loss of toxicity, CRM-197 is believed to adopt a similar 3-D fold as the wild-type diphtheria toxin. Part of the chemical property surrounding His-21 of the wild-type species is thus likely to be preserved in the case of CRM-197 and this should account for the chemical reactivity of His-21 and Lys-24 observed here. This proposal is further supported by two relevant observations: (a) Selective modification of His-21 of CRM-197 by S-DABIA occurs only when the protein exists in native form. When CRM-197 is reduced and carboxymethylated or in the presence of 5 M GdmCl, the reactivity of His-21 is no longer detectable. (b) His-21 of both CRM-197 and the wild-type diphtheria toxin display similar properties toward iodoacetamide modification (data not shown).

Iodoacetamide is known to react specifically with the thiol group of cysteine [19]. However, iodoacetamide also reacts with histidine, lysine and methionine residues in special cases, particularly when those amino acids are located along the active sites of proteins [20–22]. We have demonstrated in this report that His-21 of CRM-197 reacts selectively with S-DABIA, a colored iodoacetamide. Conditions have also been found that allow His-21 to be preferentially modified with one end of HPBIA (a bifunctional iodoacetamide). It is interesting to notice that while His-21 and Lys-24 could be sequentially modified and intra-bridged by HPBIA, only His-21 is reactive toward mono-functional iodoacetamide, such as S-DABIA. There are two possible explanations for this result. One is that modification of His-21 induces changes of microenvironment of Lys-24 and renders it inactive toward S-DABIA. Another explanation is that steric hindrance prevents His-21 and Lys-24 from being modified simultaneously by the bulky S-DABIA.

These findings offer a potentially useful route of preparing CRM-197 based protein-protein conjugate with higher homogeneity. We have demonstrated here that CRM-197 is able to bind to a small cysteine peptide via His-21. It remains to be shown whether HPBIA activated CRM-197 could efficiently bind to large proteins with high yield. A high degree of homogeneity may not be a prime concern for the vaccine efficacy. Limitation of the crosslinking site may also reduce the yield of protein-protein (peptide) conjugation. However, a homogeneous product will certainly facilitate the task of structural characterization and quality control that are required to ensure the consistency of producing CRM-197 based conjugates. In addition, this finding may also provide a new route to attaching a functional ligand to the catalytic domain of the diphtheria toxin.

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