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FOR THE RECORD

Crystal structure of heat-labile enterotoxin from *Escherichia coli* with increased thermostability introduced by an engineered disulfide bond in the A subunit

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Abstract: Cholera toxin (CT) produced by *Vibrio cholerae* and heat-labile enterotoxin (LT-I), produced by enterotoxigenic *Escherichia coli*, are AB_5 heterohexamers with an ADP-ribosylating A subunit and a G_{M1} receptor binding B pentamer. These toxins are among the most potent mucosal adjuvants known and, hence, are of interest both for the development of anti-diarrheal vaccines against cholera or enterotoxigenic *Escherichia coli* diarrhea and also for vaccines in general. However, the A subunits of CT and LT-I are known to be relatively temperature sensitive. To improve the thermostability of LT-I an additional disulfide bond was introduced in the A1 subunit by means of the double mutation N40C and G166C. The crystal structure of this double mutant of LT-I has been determined to 2.0 Å resolution. The protein structure of the N40C/G166C double mutant is very similar to the native structure except for a few local shifts near the new disulfide bond. The introduction of this additional disulfide bond increases the thermal stability of the A subunit of LT-I by 6 °C. The enhancement in thermostability could make this disulfide bond variant of LT-I of considerable interest for the design of enterotoxin-based vaccines.

Keywords: cholera toxin; disulfide bond; heat-labile enterotoxin; protein engineering, thermostability; X-ray crystallography

Heat-labile enterotoxin from *Escherichia coli* (LT-I) is very similar in sequence and structure to cholera toxin (CT) secreted by *Vibrio cholerae* (Hol et al., 1995; Merritt et al., 1996). Both toxins consist of a B pentamer and a catalytic A subunit. The first step in their mode of action involves G_{M1} receptor binding by the B pentamer at the surface of epithelial cells of the small intestine. Subsequently, the holotoxin is internalized into vesicles and the single naturally occurring disulfide bond in the A subunit becomes reduced. This is followed by vesicular trafficking of the holotoxin to the Golgi and subsequent retrograde transport of the A subunit to the endoplasmic reticulum (Lencer et al., 1995; Bastiaens et al., 1996). In an as-yet unknown manner, the A subunit is translocated across the membrane to reach the cytosol, where it then activates $G_{s\alpha}$ by ADP-ribosylation of Arg201 (Robinshaw et al., 1986). For full activity the A1 and A2 domains of the A subunit must be separated by proteolytic cleavage and Cys187–Cys199 disulfide bond reduction (Gill, 1976; Mekalanos et al., 1979; Moss et al., 1981).

The crystal structures of the AB_5 holotoxin forms of LT-I (Sixma et al., 1991, 1993) and CT (Zhang et al., 1995) have revealed many molecular details about the latent structure of these toxins. Nevertheless, structural information concerning the mechanism of activation as well as the membrane translocation process of the A subunit is still mostly absent. The activation mechanism is intriguing because events at the activation site near Cys187–Cys199 of CT or LT-I must affect the active site despite the fact that the two sites are separated by more than 20 Å. Proteolytic cleavage alone does not alter the conformation of the LT-I holotoxin (Merritt et al., 1994). Recently, investigations on the Arg7Lys mutant have provided some insight into the conformational changes that are likely to occur upon activation (van den Akker et al., 1995).

The importance of particular conformational changes in proteins can be investigated by introduction of movement-restricting disulfide bonds. This approach has been used successfully in several cases. For example, specific conformational changes occurring during the membrane insertion process of diphtheria toxin have been elucidated recently through introduction of constraining disulfide bonds that inhibit these local structural movements (Falnes et al., 1994; Zhan et al., 1994). Along a similar line, we designed and introduced the disulfide bond mutant N40C/G166C in the A subunit of LT-I with two different purposes in mind. The first goal is of a fundamental nature: increasing our knowledge about the mode of action of these toxins by introduction of a restraining disulfide

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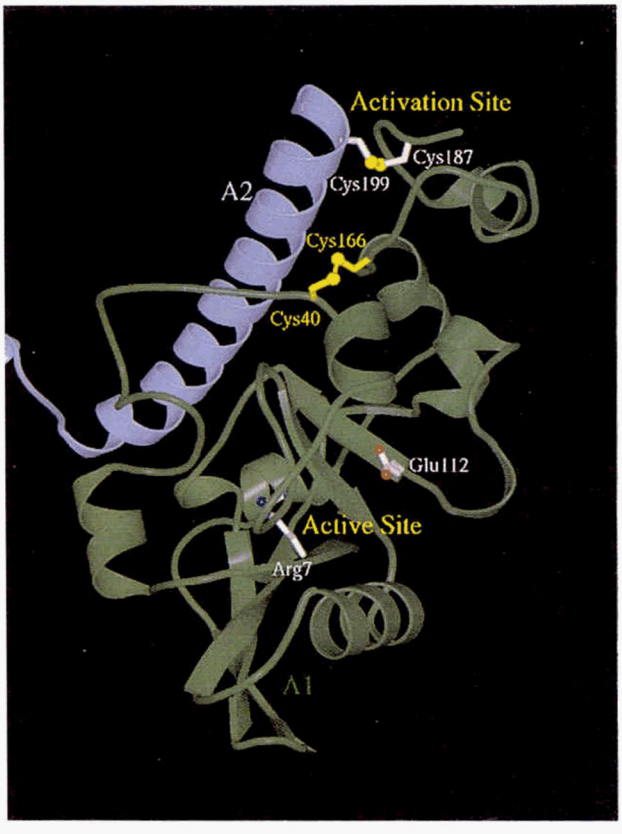


Fig. 1. Schematic diagram of the A subunit of the double cysteine mutant of LT-I. The A1 subunit is depicted in green and the A2 linker is shown in blue. In addition to the engineered disulfide bond Cys40–Cys166, the location of the activation site, including Cys187–Cys199, as well as the active site near Arg7 and the catalytic Glu112, are highlighted. Residues 189–195 of the activation "cleavage loop" are not depicted because they are not visible in the electron density map. This figure was generated using the programs MOLSCRIPT and RASTER3D (Kraulis, 1991; Merritt & Murphy, 1994).

bond, while the second addresses a more practical issue: (1) the introduction of a disulfide bond might constrain, and thereby probe, functional conformation changes during activation of the A subunit because it is located between the activation site and the catalytic active site (Fig. 1); (2) the introduction of a disulfide bond might enhance the thermostability of the LT-I holotoxin, as engineered disulfide bonds have been shown to be able to enhance the thermostability of proteins substantially (Pace et al., 1988; Matsumura & Matthews, 1991). Enhanced thermostability of cholera toxin and heat-labile enterotoxin can improve vaccines containing these holotoxins. The interest of LT-I and CT for use in vaccines arises from two different potential applications. First, a suitable vaccine against cholera and enterotoxigenic *Escherichia coli* diarrhea is still not available, even though the first cholera vaccine was used more than a century ago (Finkelstein, 1995). Cholera toxin B subunit alone added as an immunogen to the whole-cell vaccine (BS-WC vaccine) has been shown to be advantageous (Svennerholm & Holmgren, 1995). Secondly, LT-I and CT have been found to be potent stimulators of the mucosal immune response when delivered mucosally (Pierce & Gowans, 1975; Elson & Ealding, 1984) and these toxins are among the most potent mucosal adjuvants known (Holmgren et al., 1993). CT and CT-B_5 have been found to be effective as adjuvants when co-administered with a variety of different antigens (Elson, 1996) and may aid in the design of oral vaccines for immunization against a broad range of different pathogens.

The current focus of engineering vaccines against LT-I or CT is to use the complete toxin including the A subunit, preferably inactivated to eliminate its toxic activity in the gut. Recently, several groups were able to remove the toxic activity of LT or CT by site-directed mutagenesis in the A subunit while retaining the adjuvant activity of the AB_5 hetero-hexamer (Dickinson & Clements, 1995; Douce et al., 1995; de Haan et al., 1996; Rappuoli et al., 1996). One potential disadvantage of vaccines containing these toxins is that, as the name heat-labile enterotoxin implies, the AB_5 holotoxin is not thermostable. The thermostability of vaccines is of interest because most vaccines are sensitive to high temperatures and thus need to be stored in a "cold-chain," which makes vaccine delivery in Third World countries more difficult and adds to the cost. The oral BS-WC vaccine has been found to be relatively

Table 1. *Crystallographic data and refinement statistics for the LT-I N40C/G166C double mutant structure complexed to lactose*[a]

Space group	$P2_12_12_1$
Cell dimensions (Å)	119.7 101.1 64.2
Completeness cumulative to 2.0 Å (highest resolution shell)	90.5% (72.3%)
R-merge (highest resolution shell)	5.3% (17.5%)
Resolution limits in refinement	10–2.0 Å
Reflections in refinement	45,930
Reflections in test set (R_{free})	2,420
No. of atoms in model (including five lactose molecules)	6,093
No. of waters	353
R-factor	18.8%
R_{free}	26.1%
RMS deviations from ideality	
Bond distances	0.011 Å
Bond angles	1.9°

[a]The protein crystallized, using the three-layer capillary method, under very similar conditions as wild-type LT-I complexed with lactose (Sixma et al., 1992): 5 mg/mL of LT-I N40C/G166C, 5.6% PEG 6000, 100 mM Tris pH 7.5, 100 mM NaCl, 0.02% NaN_3, 75 mM lactose, 1.5 mM guanyltyramine (the latter substrate analogue could not be located in the electron density maps). Data were measured from a single crystal on an RAXIS-II image plate detector and processed using DENZO (Otwinowski, 1990). The cell dimensions were virtually identical to the wild-type LT-I + lactose structure (PDB identifier 1LTT), and those coordinates were used as a starting point for standard crystallographic refinement using X-PLOR (Brünger et al., 1987). About 5% of the reflections were used for monitoring the R_{free}. Model building was done using the program O (Jones et al., 1991). The R_{free} after rigid body refinement, which was carried out using data between 10.0–3.5 Å, was 32.5% for data to 2.0 Å. Because the starting model for refinement and the mutant structure are isomorphous, the majority of the R_{free} reflections used for the mutant refinement are biased by the earlier refinement of the wild-type structure, and the reported R_{free} value for the N40C/G166C mutant structure is, therefore, partially biased. However, about a third of the reflections, between 2.0–2.3 Å, are unbiased because the wild-type LT-I structure was determined to 2.3 Å resolution; the R_{free} improved during the refinement of the mutant structure. The enzymatic assay and cytotoxicity assay of the N40C/G166C mutant were carried out as described by Feil et al. (1996), and will be published elsewhere. Coordinates were deposited with the Brookhaven Protein Data Bank and have the PDB entry code 1LT3.

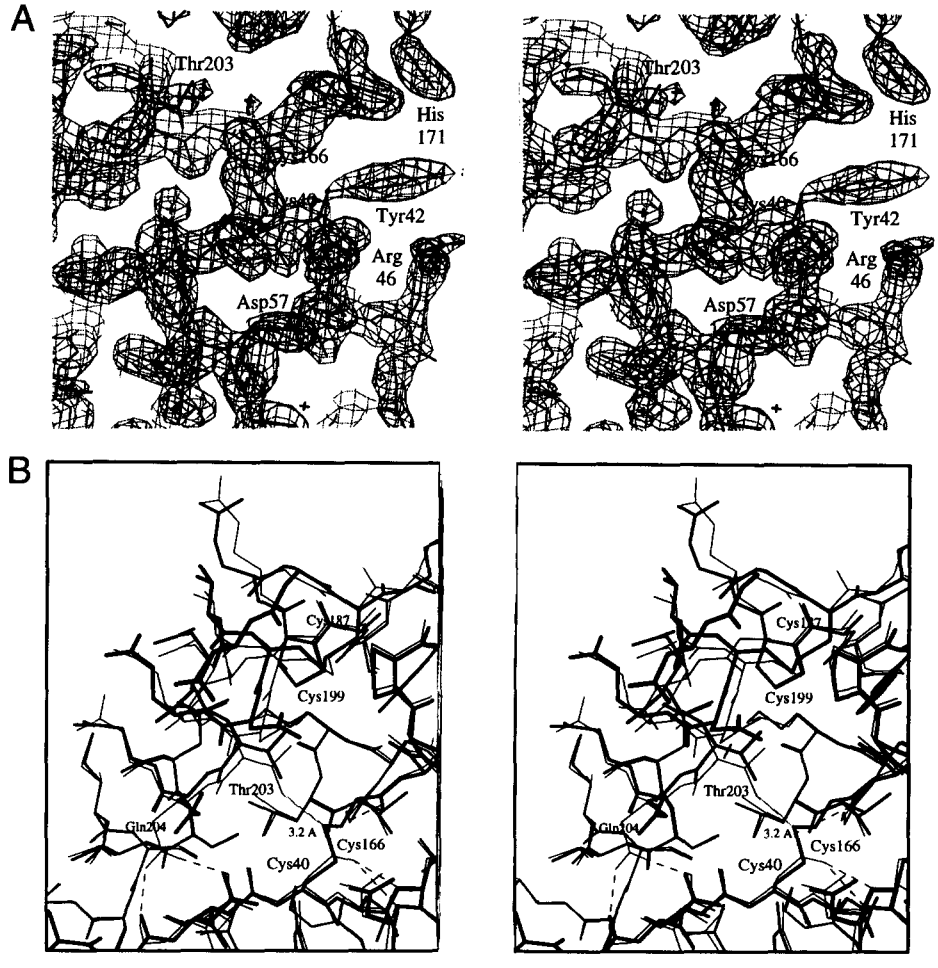
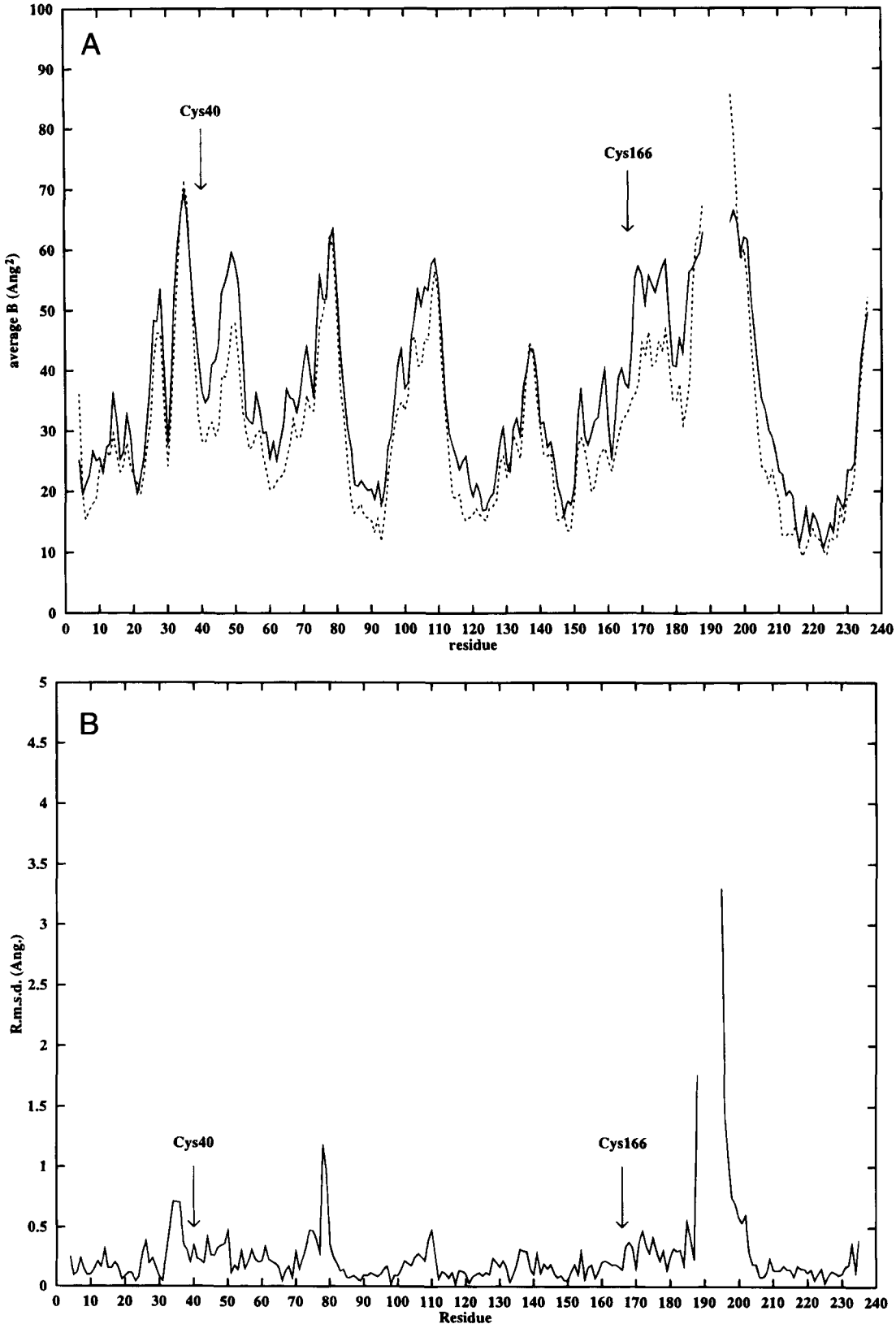


Fig. 2. A: Stereo figure showing the $2|F_o| - |F_c|$ electron density at the site of the engineered disulfide bond in the A1 subunit of LT-I. The $2|F_o| - |F_c|$ map is contoured at the 1.0 σ level. The dihedral angles of the right-handed Cys40–Cys166 disulfide bond are $\chi_1 = -175°$, $\chi_2 = 68°$, $\chi_3 = 101°$, $\chi_2' = -131°$, $\chi_1' = -160°$. The C^α-C^α distance between Cys 40 and Cys166 is 5.3 Å. Right-handed disulfide bonds are quite variable in their dihedral angle pattern but both the χ_3 angle and the C^α-C^α distance are close to values observed previously for this class of disulfide bonds (Katz & Kossiakoff, 1986). **B:** Stereo figure showing a superposition of the double mutant LT-I structure with the wild-type LT-I structure at the site of the two disulfide bonds in the A subunit. The double cysteine mutant of LT-I is shown in thick lines while the wild-type LT-I structure is depicted in thin lines. The engineered Cys40–Cys166 disulfide as well as the native disulfide Cys187–Cys199 are labeled. The hydrogen bonds made by Asn40 in the wild-type structure are shown as dashed lines as well as the hydrogen bonds of the side chain of Gln204 in the wild-type structure. This figure was generated using the programs O (Jones et al., 1991) and MOLSCRIPT (Kraulis, 1991).

stable at elevated temperatures although the CTB_5 component decreases by about 50% after 6 months at 42 °C (Ahmed et al., 1994). Vaccines containing the A subunit of CT or LT are likely the be even less thermostable than those containing the B subunit alone because the A subunit of CT denatures irreversibly at 51 °C while the B pentamer denatures at 74 °C (Goins & Freire, 1988). The introduction of an additional disulfide bond can be a first step to enhance the stability of vaccines containing the A subunit or the AB_5 holotoxin, provided that its ability to stimulate the immune response is still potent.

With the two goals listed above in mind, the position of the potential disulfide bond created by the mutations N40C and G166C

Fig. 3. (*facing page*) **A:** Plot of the average temperature factors of the main chain atoms of A subunit of N40C/G166C and wild-type LT-I + lactose (Sixma et al., 1992). The temperature factor distribution per residue of the double mutant N40C/G166C of LT-I are depicted in solid lines. The temperature factor distribution per residue of the wild-type LT-I complexed with lactose are depicted in dashed lines. The temperature factors for the newly created disulfide bond residues Cys40 and Cys166 are indicated by an arrow. **B:** Figure showing the root-mean-square deviations (RMSD) of the main chain atoms within the A subunit between the N40C/G166C variant and the wild-type LT-I both complexed with lactose (Sixma et al., 1992) structure plotted per residue. The RMSD values of the newly created disulfide bond residues Cys40 and Cys166 are indicated by an arrow. The residues 196–200 in the mutant structure deviate the most from LT-I + lactose structure, but these residues are not very well defined in the structure because the electron density was very poor for those highly disordered residues.


A
Cys40
Cys166
average B (Ang²)
residue
B
Cys40
Cys166
R.m.s.d. (Ang.)
Residue

in the A1 subunit of LT-I was chosen from a list of plausible new disulfide positions within the A subunit generated by the program SSBOND (Hazes & Dijkstra, 1988). The double mutant N40C/G166C of heat-labile enterotoxin was constructed and expressed in *E. coli* and purified similarly to the method described by Feil et al. (1996). The present study reports the 2.0 Å resolution crystal structure of the N40C/G166C double mutant of heat-labile enterotoxin.

The crystals of the LT-I N40C/G166C disulfide bond mutant complexed with lactose were isomorphous to those of the wild-type LT-I lactose complex (Sixma et al., 1992). The three-dimensional structure of the mutant could be determined to 2.0 Å (Table 1). All residues in the structure were well defined except for a few regions in the A subunit. Therefore, a number of residues of the A subunit are not included in the model; these comprise the three N-terminal, the four C-terminal residues, and residues 189–195. Residues 196–200, comprising the tip of the A2 helix, are also less well defined in the electron density but are included in the model.

The electron density at the site of the two engineered cysteines confirmed the formation of the new disulfide bond (Fig. 2a). The formation of this new disulfide bond was also evident from SDS-page gel electrophoresis experiments, which revealed an altered mobility for the N40C/G166C mutant compared to wild-type LT-I A subunit (not shown). The engineered disulfide bond is a right handed disulfide ($\chi_3 = 101°$) and is partially buried: the S^γ atom of Cys40 is only 25% solvent accessible and the S^γ of Cys166 is 45% solvent accessible. The introduction of the engineered disulfide bond does not perturb the overall structure of the A subunit as the r.m.s.d. between the native LT-I/lactose complex and the disulfide bond mutant is only 0.38 Å for all 226 Cα atoms. However, the introduction of the disulfide bond Cys40–Cys166 does cause a slight increase in temperature factors near this site (Fig. 3a) as well as a few small local shifts (Fig. 3b).

The most noticeable structural difference is a bend of the upper region of the A2 helix (Fig. 2b). However, this bend is in a region of the A2 helix with high temperature factors (Fig. 3a) and poor electron density in both the mutant and wild-type LT-I structure and might, therefore, be not a significant structural change. The slight increase in temperature factors of residues neighboring Cys40 and Cys166 (Fig. 3a) is most likely due to the loss of three stabilizing hydrogen bonds involving Asn40 in the wild-type structure, which are only partially compensated by one new hydrogen bond of the new Cys40 side chain (Fig. 2b). The three hydrogen bonds of Asn40 are made by its $N^{\delta 2}$ atom, which is located 3.0 Å from the backbone oxygen of Gly166, and its $O^{\delta 1}$ atom, which is located 3.0 Å and 3.2 Å from the backbone nitrogens of Tyr42 and Asp43, respectively. The one hydrogen bond of Cys40 in the mutant structure is made by its S^γ atom, which is 3.8 Å from the backbone nitrogen of Asp43, although this hydrogen bond is most likely not very strong because the S^γ atom is part of a disulfide bond. As mentioned above, the two sulfurs of the newly created disulfide bond remain partially exposed to solvent.

In conclusion, the introduction of the disulfide bond Cys40–Cys166 of the A subunit of LT-I does not cause major differences in the structure but is accompanied by small local shifts as well as a slight increase of thermal motion around the new disulfide bond. This observation that engineered disulfide bonds can enhance the stability of the protein without making the structure more rigid has also been observed in T4 lysozyme (Pjura et al., 1990; Jacobson et al., 1992).

The biochemical and biological properties of this mutant are somewhat different from those of the wild-type protein. The ADP-ribosylating activity determined by an in vitro assay under reducing conditions, using the artificial substrate diethylamino benzylidine-aminoguanidine (DEABAG), was less than 15% that of wild-type LT-I (Feil et al., unpubl. obs.). However, the treatment of Chinese hamster ovary cell cultures with the mutant toxin still produced about 50% of the cytotoxic effect observed for the wild-type LT-I treatment (Feil et al., unpubl. obs.). As these experiments are preliminary and have not yet been carried out with each of the single site mutants as controls, we refrain from interpreting these observations for the disulfide bond mutant at a functional level.

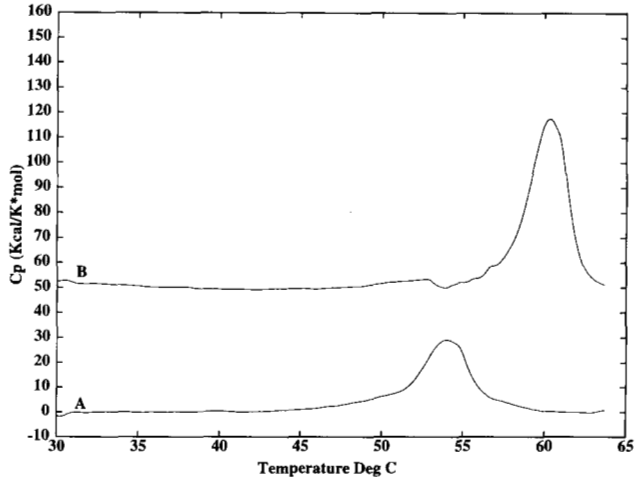


Fig. 4. Excess heat capacity function versus temperature for the A subunit of wild-type LT-I (curve A) and the N40C/G166C mutant of LT-I (curve B). The calorimetric measurements were performed following the protocol described for cholera toxin (Goins & Freire, 1988). The protein concentration in both measurements was 1.0 mg/mL and the sample volume was 0.899 mL. The calorimetric scans were performed with a Model 5100 Nano Differential Scanning Calorimeter (Calorimetry Sciences Corporation).

The thermostability of the A subunits of N40C/G166C LT-I and wild-type LT-I were measured using differential scanning calorimetry. These measurements showed that the melting temperature for the disulfide bond mutant of LT-I is 60 °C, whereas the wild-type LT-I A subunit has a melting temperature of 54 °C (Fig. 4). The introduction of an additional disulfide bond at positions 40 and 166 in the A subunit of LT-I thus increases the thermostability by 6 °C. The melting temperature of the A subunit of the engineered LT-I A subunit is also 9 °C higher than that for the A subunit of CT (Goins & Freire, 1988). The melting temperature for the B pentamers of wild-type LT-I and N40C/G166C LT-I is in both cases 86 °C (not shown).

The N40C/G166C LT-I structure shows that an increase in the thermal stability of LT-I has been achieved by introduction of a disulfide bond while causing only limited structural perturbations. This is another step forward in our structural studies to unravel the activation mechanism of the cholera toxin family at a three-dimensional level. Due to its enhanced thermostability, the mutant toxin might have implications for optimal design of cholera vaccines that are based upon the presence of the LT-I or CT holotoxin. Furthermore, introduction of this stabilizing disulfide into the A subunit may be combined with the introduction of other mutations that reduce or abolish toxicity while maintaining potent activity as a mucosal adjuvant (Dickinson & Clements, 1995; Douce et al., 1995; Rappuoli et al., 1996).

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