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Heat-labile enterotoxin crystal forms with variable A/B_5 orientation

Analysis of conformational flexibility

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A new native crystal form of heat-labile enterotoxin (LT) has two AB_5 complexes in the asymmetric unit with different orientations of the A subunit with respect to the B pentamer. Comparison with other crystal forms of LT shows that there is considerable conformational freedom for orientating the A subunit with respect to the B pentamer. The rotations of A in different crystal forms do not follow one specific axis, but most of them share a hinge point, close to the main interaction area between A and B_5. Analysis of the two high-resolution structures available shows that these rotations cause very little change in the actual interactions between A and B_5.

Heat-labile enterotoxin; Toxin; Conformational change

1. INTRODUCTION

Heat-labile enterotoxin (LT) is a major virulence factor of enterotoxigenic *Escherichia coli*, which causes diarrhoea with effects varying from mild 'traveller's diarrhoea' to dehydration and death, the latter primarily in young children in developing countries [1]. The protein shares epitopes, high homology (~80% sequence identity) and mode of action with cholera toxin (reviews [2–4]). These toxins consist of five identical B subunits (103 amino acids each), and a single A subunit (240 amino acids). The B subunits bind to G_{M1} gangliosides on the host epithelial cells allowing internalisation of the enzymatic A subunit. The A subunit is split, by reduction of a disulfide bond (187–199) and cleavage of the peptide chain into two fragments: A1 (residues 1–~192) and A2 (~193–240).

The crystal structure of LT [5] showed that the interaction of A and B is mediated mainly by the A2 fragment, which extends through a central pore in the B pentamer, with extensive hydrophobic and hydrophilic contacts. The A1 fragment, on the other hand, shares only a few salt bridges with the B pentamer (Sixma et al., manuscript in preparation). In two different crystal forms it has been noted that the A1 fragment has a flexibility with respect to the B subunit [6,7]. This flexibility could have importance during the import of the A subunit (or the A1 fragment alone) into the target cell. Here we present a fourth crystal form of LT containing two AB_5 complexes of LT in the asymmetric unit, with yet two more different orientations of the A subunit. The variability of A with respect to B_5 is analysed and a more detailed comparison of the A/B interactions is made for the two crystal forms with high resolution.

2. EXPERIMENTAL

Heat-labile enterotoxin from porcine *E. coli* was overexpressed, using plasmid EWD299 in *E. coli* C600 and purified according to Pronk et al. [8]. Crystallisation of LT in the $P2_1$ space group occurs spontaneously in TEAN (100 mM Tris, 1 mM EDTA, 0.02% azide, 200 mM NaCl), but usually results in twinned crystals, of a non-merohedral type [8]. Conditions leading to the crystals used for the structure determination, in space group $P2_12_12_1$, often give crystals in space group $P2_1$ as well. If this crystallisation is sufficiently slow it sometimes gives single or barely twinned crystals. The speed of crystallisation could be lowered by increasing the initial salt conditions, since LT crystallises under low salt conditions, but it also depends on the condition of the protein: if the protein has 'aged' [9], crystals grow more slowly. The crystals were grown at room temperature by liquid/liquid diffusion in capillaries, starting from a protein solution containing 10 mg/ml LT in 500 mM KF containing TEA (100 mM Tris, 1 mM EDTA, 0.02% azide) diluted 1:1 with a precipitant solution containing 3–6% PEG 6000 in TEA, all at pH 7.5. Before mounting, the crystal was equilibrated in a standard mother liquor (6% PEG 6000, 175 mM

Abbreviations: LT, heat-labile enterotoxin; rms, root mean square; ccf, correlation coefficient; PC, Patterson correlation.

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Table I

Crystallographic data

		Native $P2_12_12_1$ [8]	Native $P2_1$ (this study)	Samarium [6]	Lactose [7]
Space group		$P2_12_12_1$	$P2_1$	$P2_12_12_1$	$P2_12_12_1$
Cell dimensions	a-axis (Å)	119.2	84.1	118.8	119.8
	b-axis (Å)	98.2	78.8	97.5	101.2
	c-axis (Å)	64.8	123.2	65.0	64.2
	β (degrees)	90.0	99.5	90.0	90.0
Resolution (Å)		1.95	3.4	3.4	2.3
Number of measurements		230816	57087	22101	73103
Number of unique reflections		53040	13971	5779	32172
R_{merge} (%) ($\Sigma\|I-<I>\|/\Sigma<I>$)		8.8	7.1	5.8	6.4
Completeness (%)		94.4	62.5	57.1	90.9
Completeness to 4.1 Å (%)		89.9	83.8	81.5	93.8
R-factor (%) ($\Sigma\|F_o-F_c\|/\Sigma F_o$)		18.2	26.2*	33.7*	16.8

§ Synchrotron data collection at the EMBL outstation in Hamburg, on image plate with Hendrix–Lentfer scanner; Sixma et al., manuscript in preparation.

*Only rigid body refinement, starting from native $P2_12_12_1$ structure.

KF in TEA, pH 7.5). These crystals have two AB_5 molecules in the asymmetric unit (V_m = 2.37 Da/Å^3, solvent content ~51.9%).

Data were collected from a single crystal at room temperature on an Enraf Nonius FAST area detector, using an Elliot GX 21 rotating anode as X-ray source (Table I), processed using MADNES [10], and scaled and merged using programs of the local BIOMOL program system. Twinning of the crystal was checked by precession photography afterwards, but no twinning was detected.

The structure was solved by molecular replacement using X-PLOR with PC-refinement, optimisation of the standard linear correlation coefficient between $|E_{obs}|^2$ and $|E_{model}|^2$ [11]. The A and B-subunits were treated separately in this procedure. The high resolution $P2_12_12_1$ native structure [5] (Sixma et al., manuscript in preparation) was used as a model. Rigid body refinement was performed as implemented in X-PLOR, initially with each AB_5 complex as a group, and followed with 16 groups, eight for each AB_5 complex (5 B subunits, A1, and A2 split into two fragments comprising residues 196–222 and 223–240, respectively). No further refinement was done because of the limited resolution.

Analysis of the rotational variation was done by superimposing structures by the method of Rao and Rossmann [13], followed by the method of Kabsch [14]. The native high-resolution structure was used as reference molecule: in step 1 the B pentamers of the other crystal forms were superimposed on the B pentamer of the native high resolution structure. In the next step the resulting coordinates were the starting point for superposition of A1 subunits. The rotation obtained in the second step relates to the difference in relative orientation between A and B.

3. RESULTS

The structure of LT in crystal form $P2_1$ has been solved using X-PLOR molecular replacement with PC refinement, using separate steps for the A and B subunits. PC refinement [11] resulted in a very clear signal for the B pentamers (Ccf: 18.7% and 16.5%, respectively for three out of the five possible orientations of each pentamer). One A subunit also had a very clear signal (Ccf: 8.4%); the second A subunit was found as the next highest peak, but this was barely above the noise (Ccf: 4.4%). The orientations of the A subunits agreed within a few degrees each with one of the five possible rotations for a B pentamer. Translation functions were very clear for the B pentamers, and positions for the B pentamers were used as starting positions for the rigid body refinement. Rigid body refinement of the structure, with X-PLOR, resulted in a crystallographic R-factor of 26.2% (Table I). Two checks were made for the correctness of the position of the A subunits: (1) structure factors calculated leaving out one A subunit, gave R values ($\Sigma|F_o-F_c|/\Sigma F_o$) for the remaining model of 32.9% (omitting the A subunit of $P2_1$-I) and 32.5% (omitting the A

Table II

Relative rotations (degrees) of A subunits (A1 fragment) with respect to B pentamer in heat-labile enterotoxin from *E. coli*. These rotations were obtained by first superimposing the B pentamers and then determining the rotations of the A1 fragment in the left column with respect to the A1 fragment in the heading of each column. The polar rotation angles, κ, ϕ, ψ, define an angle κ around an axis defined by ϕ (deviation from x in the x–z plane) and ψ (deviation from y) according to the convention of Rossman and Blow [16]

	Native $P2_12_12_1$			$Sm(NO_3)_3$			Lactose			Native $P2_1$-I		
	κ	ϕ	ψ	κ	ϕ	ψ	κ	ϕ	ψ	κ	ϕ	ψ
Native $P2_1$-I	7.5	26.4	77.8	6.0	38.1	84.8	5.6	34.0	120.5	2.7	55.8	60.8
Native $P2_1$-II	5.5	14.9	87.8	3.9	29.2	101.2	5.0	18.2	146.5			
Lactose	5.2	7.2	31.6	3.7	48.7	20.8						
$Sm(NO_3)_3$	2.2	344.2	64.5									

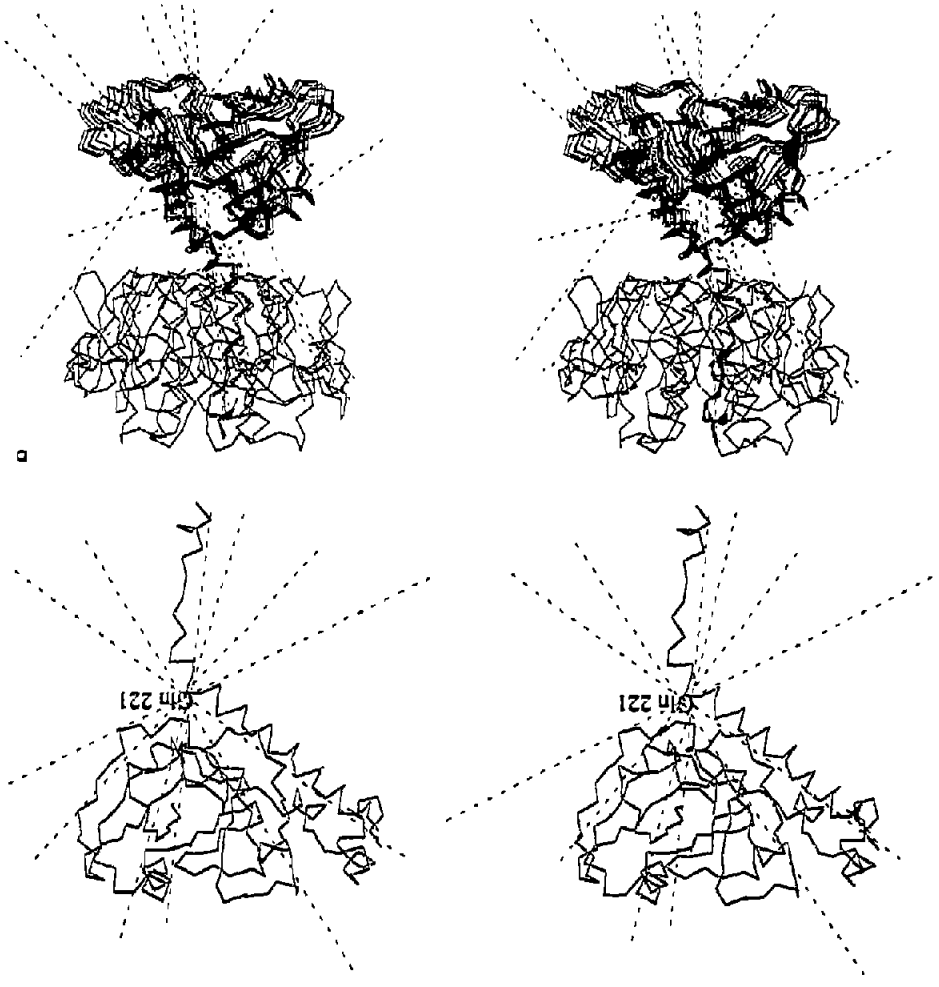

Fig. 1. Relative flexibility of the LT subunit interactions. (a) AB_5 complexes of five different structures have been superimposed with their B pentamers on the LT-native high resolution structure. The A subunits are shown, plus the B pentamer of the native $P2_12_12_1$ structure. The rotation axes which relate all A subunits are shown in dotted lines. These ten axes correspond with those in Table II. The five structures are: (i) native $P2_12_12_1$, (ii) LT:lactose complex, obtained by cocrystallisation, (iii) LT:Sm, obtained by soaking with $Sm(NO_3)_3$, (iv) native $P2_1$-I, (v) native $P2_1$-II. (b) The six rotation axes relating the A subunits of the different structures in (a), omitting the four axes related to the $Sm(NO_3)_3$ soaking structure, which are of a different type (see text). In addition the Cα trace of the A subunit of LT in the native $P2_12_12_1$ crystal form is shown. Note that all axes cross each other approximately around Gln A2:221, and that they describe a sort of 'swinging' motion around the fivefold axis.

subunit of $P2_1$-II), considerably higher than the 26.2% found for the complete model (Table I); (2) electron densities calculated with phases from a model where one A subunit was left out gave convincing density for each of the two A subunits.

Superposition studies show that both complexes in the $P2_1$ crystal form have an orientation of the A subunit with respect to the B pentamer which differs from all previously determined crystal forms of LT (Table II). There is also a difference between the orientation of the A subunits in the two complexes in the asymmetric unit, by 2.7 degrees (Table II). As in previous studies the A2 region has a hinge function: residues A1:196–222 moves with A1, and residues A2:222–237 with B_5.

Heat-labile enterotoxin has now been determined in five different forms: (i) the native structure in crystal form $P2_12_12_1$ at 2.3 Å [5] and 1.95 Å (Sixma et al., manuscript in preparation), (ii) the structure after soaking with $Sm(NO_3)_3$ [6], (iii) the structure co-crystallised with lactose [7] and (iv) the $P2_1$-I and (v) the $P2_1$-II conformations, described in this paper. A superposition of the five different crystal structures is given in Fig. 1, together with the rotation axes relating the A subunits to the different crystal forms after superimposing the B pentamer. Angular values of the rotations between the different crystal forms are given in Table II. The deviations are not large, varying between two and seven degrees, but it is clear that there is not one standard orientation of the A subunit of LT with respect to its B pentamer. The most extreme variation occurs between the native high resolution structure and the $P2_1$-I structure, indicating that the effect is independent of any outside influence other than crystal contacts, since these crystals grow under identical circumstances. Most axes

relating the variable orientations of the A subunit cross each other approximately at a common point, near Gln-221 (Fig. 1b), close to the area where most interactions between A and B occur. The exception is for axes related to the samarium nitrate soaking structure (Table II, Fig. 1). This crystal form, however, is special in two ways: (i) it was the result of a soaking experiment, and the rotational freedom has been severely limited by crystal contacts (the crystal was cracked by the soaking [6]); (ii) there are two samarium ions bound in the A/B interface, which presumably influence the relative orientation of A versus the B pentamer.

A closer comparison of the variation of the interactions is made for the two refined structures with high resolution, the native $P2_12_12_1$ and the lactose:LT complex. Fig. 2a shows the rms difference between the A subunits of these two structures, after superposition of the B pentamer. The regions of low rms deviations in A1, around residues A1:86–93, 117–128, 142–153, A1:121–123, A1:138–150, are relatively close to the B subunit, showing that this is where the hinge region is. Interactions of the B pentamer and A1 are mainly given by residues A1:33, 148 and 151. Especially the last two have a very small rms difference in the two structures, while the loop 30–33 which is in contact with A1, A2 and the B pentamer, has a slightly larger change in conformation. Other regions of A1 with low variability are in contact with A2: residue A1:93 and 95, as well as residue 123. In Fig. 2b the variable movement of the A2 fragment is clearly visible. The N-terminal fragment of A2 (A2:196–215) rotates with A1, while the C-terminal fragment (A2:223–237) follows the B pentamer, with the region 215–222 in an intermediate position. These small rms differences show that the hinge area of the rotation is in the region where the A2 helix enters the pore in the B pentamer, the place where the interaction between the subunits is largest ([5], Sixma et al., manuscript in preparation).

Specific changes of interactions are very limited: some of the salt bridges at the entrance of the pore have been slightly changed, but the overall interactions are very much the same. The solvent-accessible surface, buried by the interaction A and B is almost identical [15]. The major difference is Arg A1:143, which has a different conformation. This results in two extra A–B salt bridges and an increase of 80 Å^2 in the buried accessible surface of the lactose complex. However, the density for this residue is weak in the native structure, making this difference not very significant.

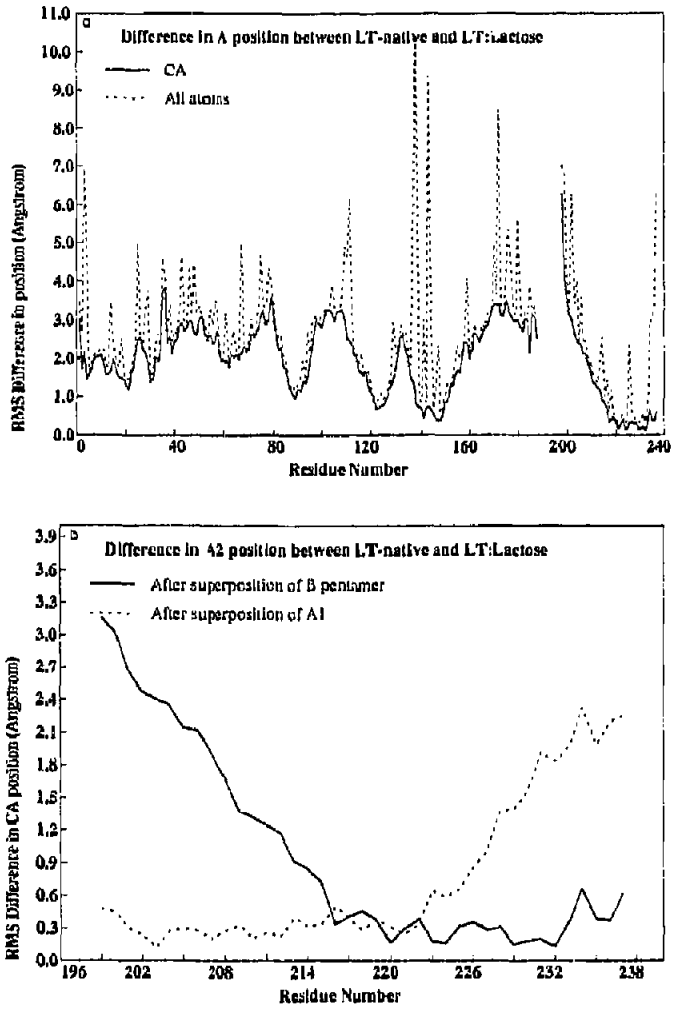

Fig. 2. Rms positional difference of the residues of the A subunits between the high resolution native LT ($P2_12_12_1$ crystal form [5], Sixma et al., manuscript in preparation) and LT:lactose complex [7]. (a) Difference after superposition of B pentamers. Thick lines indicate rms for Cα's, dashed lines for side chains (average per subunit). Residues with small differences are A:B_5 contact residues as discussed in the text. The C-terminal residues from 218 onwards show small differences because they move together with the B pentamer. Residues 189–196 are omitted because they are virtually invisible in the electron density maps. (b) Differences in Cα positions for A2 fragment, straight lines: after superposition of B pentamer; dashed lines: after superposition of A1 fragment.

4. DISCUSSION

Analysis of the new crystal form of LT was undertaken to investigate the variability in subunit interactions in the native structure. The flexibility of A with respect to the B pentamer is not significantly larger than in previously known crystal structures. Although the various rotations do not follow a common axis, they seem to have a common hinge point: in the region where A1 interacts with B_5, near residue Gln-221. The resulting changes in the interactions of A and B are minimal, because there is only a very localised interaction between A and B_5.

An interesting question is whether the observed flexibility is the unintentional result or the actual goal of these minimal contacts: having the A subunit in such an exposed position may be useful for further interactions, e.g. with membrane or other proteins, during its path from the periplasm of the parent bacteria to the cyto-

plasm of the host target cell. In this case the flexibility would only be the consequence of this exposed position of the A subunit. Alternatively, it could be that the flexibility of the A subunit itself is crucial for some step during the internalisation process. In fact it may well be that the flexibility, observed in the crystal structures elucidated so far, is only the beginning of a more elaborate conformational change taking place during the membrane translocation process.

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