Crystal Structure of the B Subunit of Escherichia coli Heat-labile Enterotoxin Carrying Peptides with Anti-herpes Simplex Virus Type 1 Activity*

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Two chimeric proteins, consisting of the B subunit of Escherichia coli heat-labile enterotoxin with different peptides fused to the COOH-terminal ends, have been crystallized and their three-dimensional structure determined. The two extensions correspond to (a) a nonapeptide representing the COOH-terminal sequence of the small subunit of herpes simplex virus type 1 ribonucleotide reductase and (b) a 27-amino acid long peptide, corresponding to the COOH-terminal end of the catalytic subunit (POL) of DNA polymerase from the same virus. Both proteins crystallize in the P43212 space group with one pentameric molecule per asymmetric unit, corresponding to a solvent content of about 75%. The overall conformation of the B subunit pentamer in the two chimeric proteins, which consists of five identical polypeptide chains, is very similar to that in the native AB complex and conforms strictly to 5-fold symmetry. On the contrary, the peptide extensions are essentially disordered: in the case of the nonapeptide, only 5 and 6 amino acids were, respectively, positioned in two monomers, while in the other three only 2 residues are ordered. The extension is fully confined to the surface of the pentamer opposite to the face that interacts with the membrane and consequently it does not interfere with the ability of the B subunit to interact with membrane receptors. Moreover, the conformational flexibility of the two peptide extensions could be correlated to their propensity for proteolytic processing and consequent release of a biologically active molecule into cultured cells.

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Atomic coordinates and structure factors (codes ILTR and I h44) of the EtxB-R2 and EtxB-Pol models have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

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§ The abbreviations used are: Etx, heat-labile enterotoxin; HSV-1, herpes simplex virus type 1; GM1, Galβ1–3GalNAcβ1–6(NeuAcα2–3)Galβ1–6Glcε1–1ceramide.
choler toxin B subunit (23), and the structurally related E. coli
verotoxin-1 (24) have also been published.

In this paper, the crystal structures of the EtxB-R2 and
EtxB-Pol chimeric proteins at 3-Å and 3.3-Å resolution, re-
spectively, are described. Moreover, structural characteristics
are correlated with intracellular processing of chimeras and
with release of biologically active peptides.

MATERIALS AND METHODS

Proteins—The hybrid EtxB-R2 protein, encoded by plasmid pAM320,
was obtained by the genetic fusion of the nine COOH-terminal amino
acids (YAGAVVNDE in single letter code) of the subunit of HSV-1
ribonucleotide reductase to the etxB gene, as described elsewhere (5).

The EtxB-Pol fusion protein, encoded by plasmid pAL3, was created
by amplifying the region of pE30 (25) corresponding to the 27 COOH-
terminal amino acids of HSV-1 POL using the polymerase chain reaction
and then inserting this fragment after the etxB gene, as reported (10).

Both EtxB-R2 and EtxB-Pol were expressed in marine non-toxino-
genic Vibrio sp. 60 (strain MTV606, obtained from Dr. A. Ichige, Uni-
versity of Tokyo), and purified as reported previously (10, 26), with
minor modifications.

Mass Determination—50 μg of purified EtxB-R2 protein was de-
salted with a Supelcosil LC318 analytical C18 column, using a gradient
of 20–60% ACN (CN 100%/5% trifluoroacetic acids in 20 mM CN). Mass
spectra were measured with an electrospray ionization spectrometer (Perkin-
Elmer Sciex API-1), on the sample directly eluted from the reverse
phase column. Mass scans were accumulated in positive mode, with
ionization and orifice voltages of 5000 V and 90 V, respectively, and a
resolution of 0.1 mass units, in the 1200–2400 mass range. The mass
peak reconstuct was obtained via the Fenn method (PE-Sciex, API-1
user manual).

Immunofluorescence and Microscopy—Cells for immunofluorescence
were plated into 24-wells trays at a density of 6 × 10⁴ cells per well, containing
one coverslip per well and grown for 24 h. After treatment with
toxin, cells were fixed with 3% paraformaldehyde in phosphate-
buffered saline for 20 min at room temperature, treated with 0.27%
NH₄Cl, 0.38% glycerine for 10 min, and permeabilized with 0.2% nico-
mine, 0.5% bovine serum albumin in phosphate-buffered saline for 30
min. Primary antibodies were diluted in the permeabilization medium
and applied to cells for 1 h. The monoclonal antibody 118-8 anti-EtxB
was kindly provided by Drs. E. Lundgren and H. Persson (University of
Umea, Sweden), whereas the polyclonal antibody anti-EtxB was ob-
ained from Dr. M. Pizza (27). The polyclonal antisemum 113 was raised
against the COOH-terminal 15 residues of POL (28), mAb 8746
(supplied by Hilka Lankinen, Institute of Virology, Glasgow) was raised
against the YAGAVVNDE peptide. After several washes, Texas
Red- or fluoresceine-conjugated secondary antibodies (from Jackson
Laboratories) were added, incubated for a further 30 min in the same medium, and then
washed. Samples were mounted in 90% glycerol, 0.2% N-propyl-galacto-
side in phosphate-buffered saline, and observed by fluorescence microscopy.
Fluorescent optical sections (1 μm thick) of cells were obtained with a
Zeiss Axioplan TV-100 fluorescence microscope, equipped with a CD
camera, using the Metamorph deconvolution software.

Crystalization—Crystals of EtxB-R2 and EtxB-Pol were obtained
with the hanging drop method. 2 μl of protein solution, approximately
7 mg/ml, were mixed with the same volume of the precipitant solution
containing 0.8 M lithium sulfate and 2% PEG 8000. The drops were
equilibrated against 0.5 ml of the same precipitant solution. Needle
crystals did not diffract under a conventional x-ray source. Data were
collected at the ELETTRA synchrotron at Trieste (Italy), using an imaging plate detector system (MAR Research), with a diam-
eter of 300 mm. Immediately before data collection the crystal was
dipped up with a loop, dipped for a very short time in a cryoprotectant solution, obtained by mixing 67% of the precipitant solution with 33%
glycerol (v/v), and frozen in a nitrogen vapor stream at 100 K. The
temperature was controlled by an Oxford Cryosystems Cryostream.

Data were processed with MOSFLM (30). Statistics on data collection
and processing are reported in Table I. The structure of EtxB-R2 was
solved with the molecular replacement technique and the AMoRe soft-
ware (31) from the CCP4 suite (32), using as a template the coordinates
of the B subunit of porcine E. coli heat-labile enterotoxin extracted from
the ABs complex (1LTS, Ref. 14). The first five maxima of the rotation
function, all related by a 5-fold symmetry, presented correlation coeffi-
cients between 13.7 and 11.6. The difference to the subsequent maxi-
mum of 11.3 was small. The highest peak of the translation function
responded to a correlation factor of 62 and to a crystallographic R
factor of 0.37. Despite an accurate search, it was impossible to fit a
second pentameric molecule in the crystal cell. Visual inspection of the
only structure solution obtained showed a reasonable three-dimen-
sional packing, despite large holes present in the crystal lattice, and no
clashes with symmetry-related molecules (Fig. 1). The structure was
subjected to a rigid-body refinement procedure that brought the crys-
tallographic R factor to 0.34. From this point onwards, visual inspec-
tions of the electron density maps and manual rebuilding using the
TOM program (33) were alternated with automatic minimizations, for
a total of 43 macromolecules. Differences in the electron density could be seen
already in the first few cycles, in correspondence to residues 13 (His
in human and Arg in porcine strain E. coli EtxB) and 46 (Ala instead of
Glu). The other 2 amino acids that differ in the human and porcine
strain sequences (Ser instead of Thr in position 4 and Glu instead of Lys
in position 102), could not be unambiguously distinguished at this
resolution. Moreover, some density was also visible close to the COOH-
terminal end of each monomeric chain, corresponding to the peptide
extension, but was difficult to interpret in terms of the known amino
acid sequence. In the course of the refinement this density became
clearer and some, but not all, residues of the extension could be fitted
into electron density maps and manual rebuilding using the
X-PLOR program (34), with different weights. The
R free (0.25). An
increase in the R crystal-detector correlation factor of R 0.12 (R free = 0.25).

During the positional and thermal factor refinement, performed with the
X-PLOR program (34, 35), no-crystallographic symmetry was im-
posed to main chain and side chain atoms, with different weights. The
atoms corresponding to the extension were excluded from this sym-
metry and allowed to refine independently. They were assigned an occu-
pancy factor of 0.75.

The molecular model determined for EtxB-R2, deprived of the amino
acids corresponding to the peptide extension and of the solvent mole-
cules, was used as the starting point for the refinement of the EtxB-Pol
structure. 32 cycles of minimization performed with the X-PLOR
program, alternated with inspections of the electron density maps and
manual rebuilding, brought the R factor to 0.21 (R free = 0.25).

Despite their reasonable size, despite the fact that the
R factor to 0.21 (R free = 0.25).

Table I

|             | EtxB-R2 | EtxB-Pol |
|-------------|---------|----------|
| λ           | 1.4 Å   | 1.0 Å    |
| Distance crystal-detector | 300 mm | 427 mm |
| α           | 1°      | 1°       |
| No. of frames | 70     | 43       |
| No. of reflections | 97277  | 64075   |
| No. of unique reflections | 27477 | 16377   |
| Resolution | 23.2–3.0 Å | 34.6–3.3 Å |
| Completeness | 97.5%  | 74.2%    |
| Rmerge      | 0.041   | 0.141    |

Dmin No. of independent reflections Completeness Multiplicity Rmerge <I/σ(I)> |
|---------|--------|----------|-----------|--------|
| EtxB-R2 |       |          |           |        |
| 6.57    | 2601   | 89.4     | 3.1       | 0.043  |
| 4.74    | 4683   | 99.8     | 3.6       | 0.035  |
| 3.90    | 5912   | 100.0    | 3.7       | 0.035  |
| 3.39    | 6890   | 100.0    | 3.7       | 0.045  |
| 3.04    | 7391   | 95.6     | 3.4       | 0.073  |

EtxB-Pol

| 7.36    | 1473   | 69.0     | 3.8       | 0.049  |
| 5.21    | 2656   | 72.4     | 4.1       | 0.100  |
| 4.26    | 3450   | 74.1     | 3.9       | 0.119  |
| 3.69    | 4070   | 74.5     | 3.8       | 0.235  |
| 3.30    | 4728   | 76.6     | 3.9       | 0.484  |

The molecular model determined for EtxB-R2, deprived of the amino
acids corresponding to the peptide extension and of the solvent mole-
nules, was used as the starting point for the refinement of the EtxB-Pol
structure. 32 cycles of minimization performed with the X-PLOR
program, alternated with inspections of the electron density maps and
manual rebuilding, brought the R factor to 0.21 (R free = 0.25).

An examination of the electron density maps, however, allowed positioning
of only a few ordered amino acids. Statistics on the final models are reported in Table II.

RESULTS AND DISCUSSION

EtxB-R2—The final molecular model consists of 4230 protein atoms, 6 sulfate ions, and 116 water molecules; a control of the stereochemistry performed with the PROCHECK program (36) indicates that only 1 residue (0.2%) falls in the “disallowed” regions, 99.0% of the residues fall in “favored” or “allowed” regions, and 0.8% in the “generously allowed” regions. All the other indicators are also consistent with or better than those expected for a structure at 3-Å resolution. The only areas that are not well ordered are loop residues 54–61. A special case is represented by the peptide extension, which is discussed in detail below.

The five polypeptide chains that form the pentamer, numbered in our model from D to H (Fig. 2), assume quite a similar conformation and are related by a 5-fold non-crystallographic symmetry axis. A restraint on the non-crystallographic symmetry was imposed during the refinement, with the exception of the loop comprising residues 54–61, as differences were evident in the electron density map among different monomeric chains (Fig. 3), and for side chains involved in intermolecular contacts.

The overall conformation of the EtxB-R2 pentamer, schematically shown in Fig. 2, is very similar to the wild type EtxB pentamer of the porcine strain E. coli enterotoxin (14); five subunits, each consisting of two antiparallel β-sheets and two α-helices, are arranged around a central 5-fold axis. A comparison of these two pentamers gives an average root mean square deviation between our model, from residues 1 to 103, and the corresponding residues of the wild type of 0.32, 0.40, 0.44, 0.62, and 0.46 Å for monomers D to H, respectively. The only significant variation between EtxB from E. coli strains of porcine and human origin is detectable around residues 54 to 61, corresponding to a solvent exposed loop. As some differences have been reported for receptor-binding specificity and antigenic determinants (38, 39), it could be proposed that these could in part depend from the exposed loop.

The amino acid sequence of our chimeric protein corresponds to the sequence of native EtxB up to position 102. Asn103 of the original sequence is substituted by Lys in the chimera, a Leu has been added at position 104 by the cloning procedure, and amino acids 105 to 113 correspond to the attached R2 nonapeptide (see legend to Fig. 2). This extension presents common features in all monomeric chains only for residues 103–105 (Fig. 3), as the β-strand that spans residues 95–100 in the native protein extends roughly to residue 105 in the chimera. The side chains of Lys103 and Tyr105 point toward the interior of the molecule, and Leu104 toward the solvent. The substitution of a polar amino acid (Asn) in position 103 with a positively charged Lys introduces a peculiar feature in the molecule: a
peak of electron density, strictly obeying the molecular 5-fold symmetry, is present close to N\textsubscript{z} of Lys\textsuperscript{103} and to N of Lys\textsuperscript{81}. Owing to its size and to the presence of the two positive charges, it has been interpreted as a sulfate ion, due to the presence of lithium sulfate in the crystal mother liquid. We can suppose that in solution these two positive charges will be neutralized by some other negative ion. From residue 105 onwards, the situation differs in all chains. In monomers E, G, and H the remaining portion of the polypeptide could not be positioned in our model. In monomer E a large and confused density can actually be seen close to the COOH terminus and to the symmetry related molecule, but there is a gap between residue 105 and this density. For this reason, we preferred not to interpret this portion. On the contrary, in monomer D amino acids 104–108 could be positioned in the electron density map: residues 105 and 106 form a sort of tight turn, so that residues 107 and 108 continue in the reverse direction with respect to the preceding \(b\)-strand. In monomer F, the polypeptide chain is ordered up to residue 109, but in this case the final part does not make a reverse turn and can better be described as an extended chain. The reason of the different behavior of the COOH-terminal residues of the latter two chains can be found in the fact that the final part of the extension in monomer F makes close contacts with a symmetry-related pentamer.

In conclusion the entire peptide extension cannot be clearly distinguished in any of the five monomers of the chimera, and the most likely explanation is that only a few residues in the extensions are ordered in the pentamer. The eventuality that portions of the COOH-terminal chains were absent from the crystal structure due to proteolytic degradation during the purification process was excluded on the basis of mass spectrometric results. These demonstrated the presence of a single species with mass 12729.5 Da, corresponding to the theoretical mass (12730.6 Da) of an EtxB-R2 monomeric chain (Fig. 5). Moreover, it would have been difficult to reconcile proteolytic cleavage at different positions with the crystal symmetry.

An important characteristic resulting from the three-dimensional structure of the chimeric protein is that the presence of the peptide extension has no effect on the overall three-dimensional structure of the B subunit pentamer. In fact, the structure of the chimera resembles that of the B subunit pentamer bound to the A subunit in the native protein. Moreover, the extension is fully confined to the surface of the pentamer opposite to the face that interacts with the membrane (14). Thus, peptide chains that extend from the COOH-terminal end of the protein should not interfere with the ability of the B subunit...
pentamer to interact with membrane gangliosides, as previously suggested by enzyme-linked immunosorbent assay experiments with soluble GM1 (40).

**EtxB-Pol**—The data for the EtxB-Pol structure were obtained only at 3.3-Å resolution and the crystal gave a weaker diffraction pattern than that of EtxB-R2, resulting in a much smaller data set. Therefore only the main features will be described. No sulfate ions could be detected and solvent molecules were not included in the model, which consists of 4191 non-hydrogen atoms. No more than 3–4 residues for each chain, corresponding to amino acids 104–107, could be safely fitted in the electron density maps. Some other electron density was visible, but not connected to the main chain and not clearly interpretable. In this case also, the B subunit pentamer maintains the doughnut-shaped structure of wild type EtxB, even if the attached POL peptide represents more than one-fourth the length of each B monomer. The 36 COOH-terminal amino acids of POL have been predicted to adopt a structure consisting of two

![Electrospray mass spectrum](A) of the EtxB-R2 monomer. The different peaks represent the indicated charge states of the molecule. The measured molecular weight is shown in the reconstructed spectrum (B).

**FIG. 5.**

**FIG. 6.** Intracellular processing of EtxB-based fusion proteins. A, Vero cells were treated with EtxB-R2 for 10 min (panels a, b, and c) or 30 min (panels d, e, and f) and probed with a polyclonal antibody specific for EtxB (EtxB, left panels) and with the monoclonal antibody 8746, specific for R2 peptide (R2, central panels) and secondary anti-rabbit fluoresceine-conjugated or anti-mouse Texas Red-conjugated antibodies, respectively. Images were collected using a ×100 objective with a deconvoluted fluorescence microscope and superimposed (EtxB/R2, right panels). A yellow color indicates colocalization of the two domains. Arrows and arrowheads point at representative EtxB1/R21 and EtxB1/R22 compartments, respectively. B, Vero cells were treated with EtxB-Pol for 10 min (panels g, h, and i) or 1 h (panels l, m, and n) and probed with monoclonal antibody 118-8, specific for EtxB (EtxB, left panels) and with polyclonal antiserum 113, specific for POL peptide (Pol, central panels) and secondary anti-mouse fluoresceine-conjugated or anti-rabbit Texas Red-conjugated antibodies, respectively. Images were analyzed as described in A. Arrows and arrowheads point at representative EtxB1/POL1 and EtxB1/POL2 compartments, respectively.
α-helical regions interrupted by a non-helical segment (41). We cannot exclude, owing to the quite low resolution of our electron density map, that a portion of the POL-derived peptide extension in our chimera could assume a definite conformation, despite being orientationally disordered with respect to the EtxB core.

**Intracellular Processing of EtxB-R2 and EtxB-Pol**—To study the intracellular fate of the two chimeras, Vero cells were treated for different times with 10 μM EtxB-R2 or EtxB-Pol and then stained either with anti-EtxB polyclonal and anti-R2 monoclonal antibodies, for EtxB-R2, or with anti-EtxB monoclonal and anti-Pol peptide antiseraum, for EtxB-Pol. Analysis by deconvolution fluorescence microscopy showed that both EtxB-R2 and EtxB-Pol bound to cell surface receptors and were internalized in a manner resembling that of wild-type EtxB. These results confirm that addition of such peptides to the EtxB COOH terminus does not interfere with the receptor binding properties of either EtxB-based fusion protein in a cell system, and is consistent with our observations on the three-dimensional structure.

After 10 min incubation the two domains of both fusion proteins fully co-localized in punctated intracellular compartments which appeared either scattered throughout the cytoplasm or in the perinuclear area (Fig. 6, A, panels a, b, and c and B, panels g, h, and i), indicating that after internalization both EtxB-R2 and EtxB-Pol fusion proteins are initially intact. After 30 min the toxin and peptide portions of EtxB-Pol and EtxB-R2 were beginning to dissociate: in addition to EtxB-R2/13. Sixma, T. K., Pronk, S. E., Kalk, K. H., Wartna, E. S., van Zanten, B. A. M., and Stow, N. D. (1994) FEMS Microbiol. Lett. 121, 249–253.
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