Formation of disulfide bonded dimer of mutated heat-labile enterotoxin in vivo

Peter A. Hedges**, Simon J.S. Hardy*

Department of Biology, University of York, York YO1 5DD, UK

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Abstract One of the two cysteines in the B subunit of heat-labile enterotoxin has been changed to a serine by site-directed mutagenesis so that the internal disulfide bond cannot form. The mutant protein, like the wild-type protein synthesised in the presence of the reducing agent dithiothreitol, does not form pentamers in the periplasm but binds to available membranes. Binding to membranes is disrupted by chaotropic agents but not by salt. More than half the molecules of mutant protein form disulfide-bonded dimers when exported to the periplasm but no dimer is detected when the protein is exported to the medium by spheroplasts.

Key words: Heat-labile enterotoxin; Disulfide bond; Membrane association

1. Introduction

Heat-labile enterotoxin of *E. coli* is a periplasmic protein consisting of a single A subunit (26.5 kDa) inserted into a ring of five B subunits (12 kDa) [1,2]. Each monomer in the pentamer of B subunits contain an internal disulfide bond linking the cysteines at positions 9 and 86 [2]. When millimolar concentrations of the reducing agent, dithiothreitol (DTT), are added to the culture, newly synthesised B subunits are exported from the cytoplasm but pentamers do not form, although pentamers already made do not dissociate [3]. More surprising to us was our recent finding that the reduced monomers, which are hydrophilic after removal of the leader sequence, associate tightly with the membranes enclosing the periplasm, from which they can only be removed by washing with chaotropic agents [4].

To ensure that these findings were due to lack of the internal disulfide bond and not some non-specific effects of the reducing agent, we used site-specific mutagenesis to change the cysteine at position 9 to a serine. The mutant protein was exported, did not form pentamers and was bound to membranes. Unexpectedly it rapidly formed disulfide bonded dimers with such high efficiency that more than half the B subunit was in this form. The dimers, like reduced wild-type monomers, were membrane-bound.

*Corresponding author. Fax: (44) (1904) 43-2860.

**Present address: EPSRC, Polaris House, North Star Avenue, Swindon SN2 1ET, UK.

Abbreviations: DTT, dithiothreitol; IPTG, isopropylthiogalactoside.

2. Materials and methods

2.1. Bacteria

E. coli K12 G6 (his$^-$) containing pDQ5 was used in all experiments. pDQ5 is 5.7 kbp and contains the mutated gene for the B subunit of heat-labile enterotoxin (*etxB*) under the *tac* promoter, the *neo* gene, the *lacI*^{q} gene and the pBR322 origin of replication. Production of B subunit is very tightly regulated. Site-directed mutagenesis of wild-type B subunit was carried out using standard kits and following the manufacturer's instructions. After mutagenesis of the TGT cysteine codon to the TCT serine codon the entire gene was sequenced and found to be otherwise identical to the published sequence [5].

2.2. Growth and labelling of cultures

Bacteria were grown in glucose M9 medium with vitamin B1, histidine and kanamycin, at 37°C with shaking. Growth was monitored spectrophotometrically. When the A^{560} reached approximately 0.4, IPTG (final concentration 1 mM) was added to induce expression of the B subunit. After 10 min [^{35}S]methionine (5 μCi/ml, 1000 Ci·mmol) was added and after a short period a high concentration of non-radioactive methionine (50 μg/ml) was added as a chase. Samples were taken either directly into 10% trichloracetic acid, or if bacteria were to be fractionated, into tubes containing an equal volume of crushed frozen 0.6 M sucrose for rapid cooling.

2.3. Fractionation of bacteria

Labelled bacteria were centrifuged at 4°C in a microfuge. The bacterial pellet from 1 ml or 0.5 ml of culture was resuspended in 100 μl of 0.1 M Tris-Cl pH 7.6, 12.5 mM EDTA, 0.3 M sucrose. 10 μg of lysozyme were added and the bacteria were incubated for 20 min on ice. The spheroplasts were spun down at 4° for 2 min in a microfuge, and the supernatant (periplasm) was removed. The spheroplast pellet was resuspended in 100 μl of 0.1 M Tris-Cl pH 7.6, 5 mM $Mg(Ac)_2$ containing 10 μg/ml DNAse and subjected to 5 cycles of freezing and thawing after which the lysed spheroplasts were centrifuged at 4°C for 45 min in a microfuge. The supernatant (cytoplasm) was removed and the membrane pellet was resuspended in 100 μl of 0.1 M Tris-Cl pH 7.6, 5 mM Mg $(Ac)_2$.

2.4. Labelling of spheroplasts

Cultures were divided into two, and one portion was induced with IPTG. After 10 min periplasms were prepared from unlabelled cells and the spheroplasts were resuspended in prewarmed growth medium containing IPTG if the spheroplasts were from the induced culture, and 0.3 M sucrose, to give the same concentration of cells as in the original culture. After 5 min at 37°C, radioactive methionine was added followed after 2 min by a chase of non-radioactive methionine. After a further 5 min incubation the labelled spheroplasts were centrifuged at 4°C for 45 min (to ensure that the membranes of lysed spheroplasts were pelleted) in a microfuge. The supernatant containing soluble secreted proteins as well as soluble proteins from lysed spheroplasts, and the pellet containing intact spheroplasts as well as membranes from lysed spheroplasts were analysed. More lysis tended to occur for spheroplasts from induced than from uninduced bacteria during this procedure (see Fig. 3).

2.5. Washing of spheroplasts from labelled cells

Spheroplasts from a frozen pellet were resuspended in 10 mM Tris pH 7.6, 5 mM $Mg(Ac)_2$, 0.5 M sucrose. To one volume of this suspension, one volume of 4 M NaCl or 4 M guanidinium chloride was added. The mixture was incubated in ice for 5 min and then centrifuged at 4°C for 45 min in a microfuge to ensure that membranes

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from lysed spheroplasts as well as intact spheroplast would be pelleted. Supernatants and pellets were analysed separately.

2.6. Analysis of fractions

Fractions were precipitated in 10% TCA and centrifuged at room temperature for 15 min in a microfuge. The supernatant was discarded, the pellet was washed with acetone. The final pellet was redissolved in sample buffer for SDS-PAGE. This was carried out using the BioRad Mini Protean II system. The proteins were visualised by autoradiography. Radioactivity in individual bands was estimated using a Molecular Dynamic PSF Phosphorimager.

3. Results

We have previously found that when the formation of the internal disulfide bond in the B subunit of heat-labile enterotoxin is prevented by the presence of dithiothreitol (DTT) in the medium, the protein is exported through the cytoplasmic membrane, but binds to the available membranes in the periplasm, instead of remaining soluble and assembling into a pentamer [4]. This conclusion was based on experiments in which B subunit synthesised in the presence of DTT sedimented quantitatively with membranes, floated quantitatively with membranes, and was distributed across both the cytoplasmic and outer membrane peaks after equilibrium sucrose density gradient centrifugation. Furthermore B subunit remained monomeric. There was no detectable formation of B subunit pentamer in the presence of DTT, even though pentamer already assembled in the absence of DTT was not affected by the reducing agent.

To ensure that these findings were caused by the inability to form the internal disulfide bond between the cysteines at positions 9 and 86, rather than by some less specific effect of DTT we used site-directed mutagenesis to change the cysteine at position 9 in the B subunit to a serine. When this mutant protein was induced and labelled with [^{35}S]Met in the absence of DTT a new prominent radioactive protein of apparent molecular weight approximately 28,000 was observed among the total labelled proteins of the bacteria analysed by SDS-PAGE (Fig. 1) provided that there was no reducing agent in the sample buffer. If there was either DTT in the sample buffer (Fig. 1), or the bacteria were labelled in the presence of 2.5 mM DTT (not shown), the new band was not observed and there was a concomitant and quantitatively equivalent increase in the radioactivity found at the position of the monomer B subunit (Fig. 1). Although it is formally possible that the new band is a specific disulfide-bonded complex between B subunit and another small protein lacking significant quantities of methionine, it is very much more likely that the new band is a dimer formed through a disulfide bond between the cysteines at position 86 in two molecules of monomer. On maximal induction about 7% of the radioactive methionine incorporated into proteins is found in the B subunit, of which more than half is in the putative dimer. No such dimer is detectable when the protein is the wild-type form that contains both cysteines (not shown).

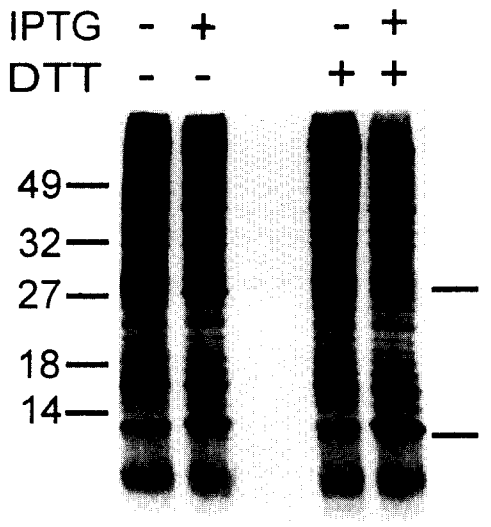

Fig. 1. Mutated B subunits form dimers. Samples of uninduced and induced cultures of G6pDQ5 that were pulsed for 15 s and chased for 5 min, are shown. The two left hand lanes had no DTT in the sample buffer. The two right hand lanes are the same samples but with 25 mM DTT in the sample buffer. Positions of monomer and dimer B subunit are indicated.

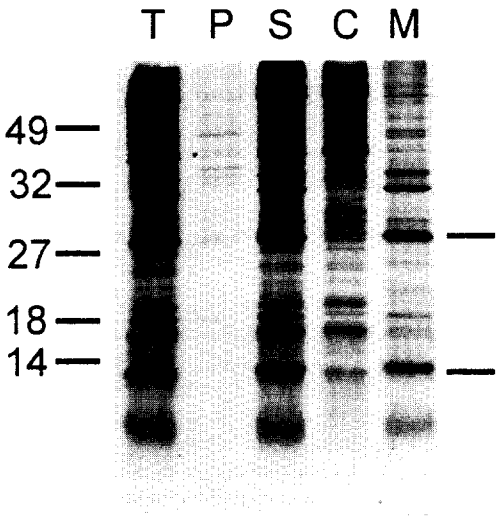

Fig. 2. Mutated B subunits fractionate with membranes. Cells labelled as in Fig. 1 were fractionated. T = total; P = periplasm; S = spheroplasts; C = cytoplasm; M = membrane.

Cell fractionation studies show that the mutant B subunit, both in its monomer and dimer forms is associated with the membranes of the bacteria. It is almost undetectable in the periplasm, and after lysis of spheroplasts sediments quantitatively with membranes (Fig. 2). Other experiments have shown that it floats quantitatively with the membranes in metrizamide gradients and that it is distributed on both the cytoplasmic and outer membranes in sucrose density gradients (not shown). In all these respects the dimer and monomer form of the mutated B subunit behave like the wild-type B subunit made in the presence of DTT. The binding to membranes is probably a consequence of the inability of the mutant protein to form pentamers in the periplasm, since other mutants of the B subunit which are unable to form pentamers are known to associate with membranes [6]. The formation of the internal disulfide bond in each monomer is therefore essential for pentamerisation and the consequent correct localisation of the toxin.

Formation of the B subunit dimer is dependent on proximity with membranes since when the mutant B subunit is made by spheroplasts where the outer membrane is not a barrier to escape into the medium, the protein remains as a monomer, present in the medium after the spheroplasts have

been removed by sedimentation (Fig. 3). However, the disulfide bond is probably not formed between monomers bound to the membrane but in the periplasm immediately after export of B subunit since conversion of monomers to dimers occurs much more slowly when monomers are first allowed to bind to membranes. The normal time course of formation of dimers is shown in Fig. 4. Dimer formation is essentially complete within 2 min of the labelling period, more than half the radioactivity in the B subunit being converted to the dimer in this time. In contrast, if the cells are labelled in the presence of DTT so that all the radioactive B subunit remains monomeric and binds to membranes, and then the reducing agent is removed, there is a very slow conversion of monomer to dimer such that only about one-fifth of the B subunits become dimers in 20 min (not shown). Meanwhile B subunits made after the removal of DTT rapidly and efficiently form dimers. In reverse experiments, dimers are only slowly converted to monomers if DTT is added after dimers are associated with membranes (not shown). It is clear therefore that once the protein is associated with membranes, formation or breakage of the disulfide bond between monomers is less readily accomplished than immediately after synthesis and export of the protein.

Association of the B subunit dimer with membranes may be disrupted by 2 M guanidinium chloride but not by 2 M sodium chloride, indicating a contribution of hydrophobic and/or hydrogen bonds to the binding (not shown).

4. Discussion

It is now clear that the inability of wild-type B subunit of heat-labile enterotoxin to form pentamers in the presence of DTT and its consequent binding to membranes are the direct effects of the reduction of the internal disulfide bond in the monomer, rather than some secondary effect of the reducing agent. These results were expected. However, an unexpected finding was that in the absence of DTT the mutant protein with a cysteine to serine substitution at position 9 should with such high efficiency and specificity form a disulfide bonded dimer between the cysteines at position 86 in each of the two monomers.

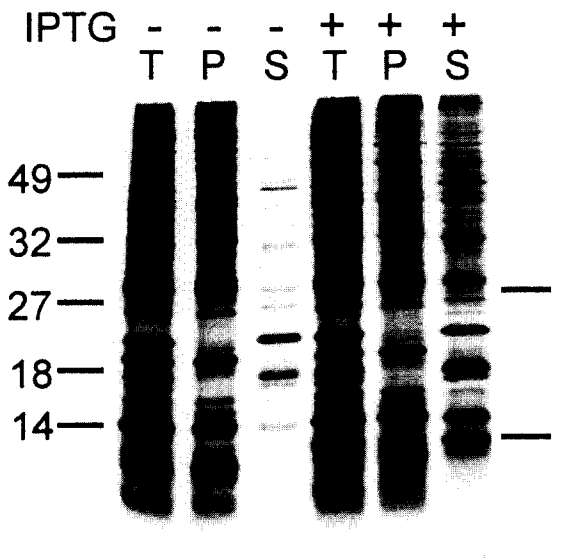

Fig. 3. B subunits secreted from spheroplasts do not dimerise or associate with membranes. Spheroplasts from induced and uninduced bacteria were labelled as described. The labelled spheroplasts were separated from labelled secreted proteins by centrifugation. The three lanes on the left show the labelled proteins from uninduced spheroplasts, the three on the right those from induced spheroplasts. T = total labelling pattern; P = pellet (retained proteins); S = supernatant (secreted proteins).

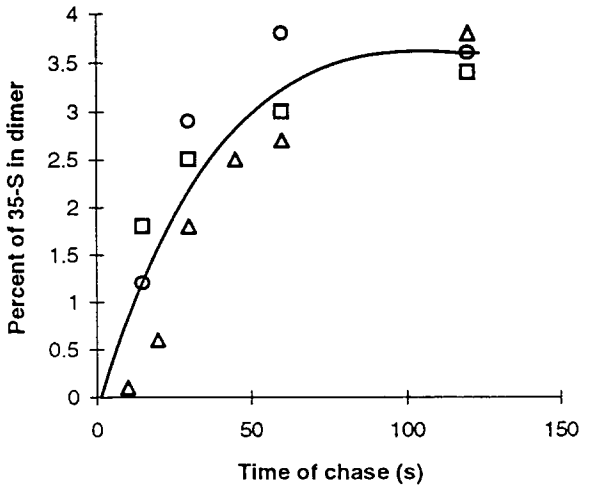

Fig. 4. Time course of formation of dimers. Cultures of G6pDQ5 were induced and after 10 min pulsed for 10 s (triangles) or 15 s (squares, circles). At various times during the chase, samples were pipetted directly into TCA (triangles, circles) or onto crushed frozen 0.6 M sucrose (squares). The radioactivity in the dimer as a percentage of the total radioactivity in proteins is shown as a function of the time elapsed since addition of chase. The percentage of the radioactivity in the equivalent section of adjacent lanes displaying the radioactive proteins from identically labelled uninduced cells have been subtracted from the unadjusted percentages to give the plotted values.

In the correctly folded B subunit monomer C86 is in a β-sheet containing β-strands 1-5-6 of one subunit and 2-3-4 of the neighboring B subunit in the pentamer [2]. C86 in β-strand 5 forms a bond with C9 that anchors a small amino-terminal α-helix on to the β-sheet. In the mutant form the bond is missing and, assuming that the secondary structure elements of the monomer can form, the α-helix will be free to move out of the way thus exposing the sulfhydryl group and making it available for formation of a disulfide bond with a similarly exposed group on another monomer. The efficiency with which the bond forms is striking and may indicate that noncovalent dimer formation is the first step in pentamer formation by the wild-type protein. However if such a dimer had a structure similar to that of a neighboring pair of B monomers in the assembled pentamer, there would be far too great a distance between the two cysteines for a disulfide bond to form. Disulfide bond formation in the mutant dimer therefore would require that the β_1-β_5-β_6 strands not involved in the 6-stranded β-sheet stabilising the dimer, should fold back over the sheet to bring the two β_5 strands close enough together for bond formation.

The B subunit of heat-labile enterotoxin is a highly charged monomer and yet forms a tight association with membranes if unable to assemble into pentamers [4,6]. The association occurs whether the B subunit is a wild-type monomer prevented from forming its internal disulfide bond, or a mutant monomer or dimer. This may be due to the interaction of the hydrophobic side of the long, highly amphipathic α helix,

spanning residues 59–78, with membrane lipids. It is unlikely to be predominently due to an electrostatic interaction with the periplasmic surface of the membranes because 2 M salt does not affect the association. Because at very early times after labelling we observe small quantities of radioactive B subunit in periplasmic preparations, and essentially none at later times, our model for the binding to membranes is that newly exported B subunit is initially free in the periplasm but soon collides with one or other of the enclosing membranes in an orientation that promotes binding. This model also explains why both monomer and dimer forms of mutant B subunit are found to be distributed over both cytoplasmic and outer membranes and why when made by spheroplasts the protein remains largely soluble. We suggest that dimerisation occurs before binding to membranes since when we allow radioactive monomers made in the presence of DTT to bind to membranes and then remove the DTT, formation of dimers is very much slower than formation of dimers from monomers synthesised after the removal of DTT. This is probably because DsbA, the enzyme implicated in forming disulfide bonds in heat-labile enterotoxin and cholera toxin is a soluble periplasmic enzyme [7,8] and does not interact as readily with proteins that are associated with membranes as with proteins of the periplasm. A lack of DsbA in spheroplasts from which the periplasm has been removed, together with the large reduction in concentration of monomers brought about by export directly into the medium rather than into the enclosed volume of the periplasm, would also explain why we saw no dimer formation in B subunit synthesised and exported by spheroplasts.

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