The hydrophobicities of cholera toxin, tetanus toxin and their components

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1. Charge-shift electrophoresis showed that cholera toxin and its subunits have no hydrophobic surfaces. 2. Amino-acid composition and sequence data suggested that the proteins have no masked hydrophobic regions. 3. The A subunit of cholera toxin may interact with polar molecules in the membrane to exert its effect inside the cell. 4. The only hydrophobic part of tetanus toxin was the H-chain.

Cholera toxin activates adenylate cyclase in most eukaryotic cells (van Heyningen, 1977; Moss & Vaughan, 1979). The molecule consists of a ring of five B subunits, and one A subunit. The B subunits (M_r about 11 500) bind specifically to the oligosaccharide moiety of ganglioside G_{M1} on the outer surface of susceptible cells. The A subunit consists of two peptides, which are joined by a disulphide bond; peptide A2 (M_r about 5000) is in close contact with the B subunits and peptide A1 (M_r about 22 000) catalyses the ADP-ribosylation of a regulatory component of the adenylate cyclase complex. As this reaction occurs inside the cell, the A1 peptide (or some part of it) must be transported across the plasma membrane.

Little is known about this transport process. Since only a few molecules of peptide A1 are probably needed inside the cell to activate the cyclase, it need not be very efficient. There is a lag phase between the initial binding of the toxin to the surface of intact cells and the subsequent increase in the cyclase activity. This may be due to the time taken for the toxin to penetrate the membrane, or to interact with membrane proteins, or for the disulphide bond in subunit A to be reduced, or for the multivalency for ganglioside G_{M1} to be satisfied, or for a combination of these factors. Photoaffinity labelling experiments show that the A1 peptide is the only part of the toxin that enters the lipid bilayer (Wisniewski & Bramhall, 1981).

In the present paper, we describe experiments showing that whole cholera toxin and both its subunits have hydrophilic exteriors and so are unlikely to interact easily with side chains of fatty acids in the membrane. Theoretical calculations

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based on the amino-acid composition and sequence of the proteins support this conclusion.

Much less is known about the action of tetanus toxin, which interacts with neuronal cells by an unknown mechanism (van Heyningen, 1980). It is made up of two chains linked by a disulphide bond. The H-chain (M_r about 100 000) binds to a ganglioside on the cell surface, but the L-chain (M_r 50 000) does not bind. Our experiments show that tetanus toxin is also hydrophilic, although the H-chain has some hydrophobicity.

Experimental

Materials

Cholera toxin was purified from culture filtrates of *Vibrio cholerae* kindly grown for us at the Centre of Applied Microbiology and Research, Porton, Wilts., U.K. The subunits were purified by chromatography on Sephadex G-75 (Pharmacia, Hounslow, Middx., U.K.) in urea at low pH (Moss *et al.*, 1976). Tetanus toxin was purified (van Heyningen, 1976) from material kindly given to us by Dr. R. O. Thomson of the Wellcome Research Laboratories, Beckenham, Kent, U.K.

Charge-shift electrophoresis

Hydrophobicity was measured experimentally by the method of Helenius & Simons (1977). The binding of the non-ionic detergent Triton X-100 (Koch-Light, Colnbrook, Bucks., U.K.) to the protein is observed by determining the charge on the protein in detergent mixtures containing Triton and another anionic or cationic detergent. Proteins with hydrophobic areas on their surface bind Triton, and so migrate in different directions in each detergent mixture when electrophoresed in 1% agarose.

Assay of cholera-toxin activity

Cholera toxin and its subunits were pre-incubated with the detergents and then assayed for the ability to activate adenylate cyclase in rat liver membranes (Tait *et al.*, 1980) and for binding to insoluble ganglioside/cerebroside mixtures (van Heyningen, 1974).

Results

Predicted hydrophobicities

There are several different methods for estimating the hydrophobicity of a protein from its amino-acid

composition (Bigelow, 1967; Barrantes, 1975), or sequence (Segrest & Feldman, 1974). The results are expressed in terms of different parameters and compared with values calculated for proteins of known hydrophobicities (Table 1). Such predictions could indicate the existence of masked hydrophobic areas that would not be detected by charge-shift electrophoresis. All the methods suggested that cholera toxin and both its subunits are probably hydrophilic, although perhaps with a little amphiphilic character especially in peptide A2. There were several hydrophobic sequences in the toxin and in the very similar A subunit of *Escherichia coli*

Table 1. *Hydrophobicities of cholera toxin and its subunits predicted from their amino-acid compositions*
The ranges of the hydrophobicity estimates for standard proteins were calculated by Barrantes (1975) and Raftery *et al.* (1975). The range quoted represents the mean $\pm$ s.d., and *n* indicates the number of proteins studied. Data on the structure of cholera toxin are reviewed by Lai (1980); 1 cal $\approx$ 4.18 J.

Protein	Estimates of parameters of hydrophobicity		
	$\bar{H}_0$ (kcal/mol) (Bigelow, 1967)	R_3 (Barrantes, 1975)	z (Barrantes, 1975)
Non-membrane proteins (<i>n</i> = 205)	0.90–1.09	0.84–1.68	–0.10 to 0.33
External membrane proteins (<i>n</i> = 24)	0.91–1.06	1.02–1.72	–0.04 to 0.28
Internal membrane proteins (<i>n</i> = 24)	1.10–1.29	0.41–0.77	0.41–0.63
Toxin			
A1 peptide	0.94–1.07	1.29	0.12–0.20
A2 peptide	0.78–0.97	1.72	–0.13 to –0.01
A subunit	0.90–1.04	1.40	0.05–0.14
B subunit	1.05	1.34	0.16–0.17
A2–(B) ₅ complex	1.02–1.04	1.39	0.13–0.15
Whole toxin [i.e. A1–A2–(B) ₅]	1.00–1.05	1.36	0.13–0.16

Table 2. *Migration of proteins on charge-shift electrophoresis*

Abbreviation: CTAB, cetyltrimethylammonium bromide. + indicates anodal migration; – indicates cathodal migration; 0 indicates no migration; * indicates that under these conditions, diphtheria toxin split into two components (see Boquet, 1979).

Detergent mixture	Direction of migration				Were hydrophobic areas detected on the protein surface?
	No detergent	Triton alone	Triton + cholate	Triton + CTAB	
Standard proteins					
Bovine serum albumin	+	+	+	+	No
Ovalbumin	+	+	+	+	No
Diphtheria toxin	+	+	+	+, –*	No
Immunoglobulin G	0	0	0	0	No
Human apoprotein B from low-density lipoprotein	+	0	+	–	Yes
Human apoprotein A1 from high-density lipoprotein	+	0	+	–	Yes
Cytochrome <i>b</i> ₅₆₁	+	0	+	Not determined	Yes
Toxins					
Whole cholera toxin	+	+	+	+	No
Subunit A	+	+	+	+	No
Subunit B	–	–	–	–	No
Whole tetanus toxin	+	+	+	+	No
H-chain	+	0	+	–	Yes
L-chain	+	+	+	+	No

heat-labile toxin (Spicer *et al.*, 1981). But the hydrophobicity indices were comparable with those found for the interiors of proteins rather than for hydrophobic proteins (Segrest & Feldman, 1974).

Charge-shift electrophoresis of cholera toxin

The charge-shift system was tested with several proteins of known hydrophobicity. Cholera toxin and both of its subunits migrated as proteins with no hydrophobic areas on their surface (Table 2).

Activity of cholera toxin

After pretreatment with the detergents used in the charge-shift experiments, cholera toxin and subunit A both retained the ability to activate the adenylate cyclase of rat liver membranes. They had similar specific activity to the native proteins. Whole toxin and subunit B both bound to ganglioside G_{M1} , insolubilized by complexing it to cerebroside, but not to cerebroside alone.

Tetanus toxin

In similar charge-shift experiments, tetanus toxin migrated as a hydrophilic protein (Table 2). Investigation of the component chains was complicated by the difficulty of preparing them pure in the absence of sodium dodecyl sulphate. Also the H-chain requires urea to keep it in solution. However, on charge-shift electrophoresis in the presence of 2-mercaptoethanol, the toxin [labelled with ^{125}I] (Greenwood *et al.*, 1963) and in the presence or absence of 4M-urea split into two components, which were identified by elution and analytical electrophoresis. The L-chain migrated with whole toxin as a hydrophilic protein, but the H-chain apparently has some hydrophobic areas on its surface.

Discussion

These experiments show that the surfaces of cholera toxin and its subunits are all hydrophilic, and the theoretical calculations suggest that there are no masked hydrophobic regions in the A1 peptide or the B subunit. If, as the calculations suggest, the A2 peptide (which is very difficult to work with experimentally after purification) is amphiphilic, its hydrophobic areas must be masked in the intact A subunit. The A2 peptide could act as a hydrophobic leader sequence for the entry of peptide A1 into the plasma membrane. This would correlate with the observation that a fluorescent derivative of A subunit showed a conformational change when toxin bound ganglioside G_{M1} (S. van Heyningen, unpublished work). But the A2 peptide is not strikingly hydrophobic and was not detected in the lipid bilayer by photoaffinity labelling (Wisniewski & Bramhall, 1981).

These results do not support suggestions that the A1 chain partitions directly into the cell membrane. But, as only very small amounts are needed inside the cell, such passage need not be a very likely event. Since A1 is hydrophilic, it could enter the membrane (where it was detected by photoaffinity labelling), after interaction with some integral membrane protein. There is evidence that the action of the toxin requires certain membrane proteins (Hagmann & Fishman, 1981). To exert its transmembrane effect, the A subunit may interact with the substrate of the toxin, other components of adenylate cyclase or protein disulphide reductase (Moss *et al.*, 1980). Alternatively the toxin may be taken up by endocytosis, but the A1 peptide would still have to cross the lysosomal membrane to activate adenylate cyclase. There is some indirect evidence that the protein does not cross this membrane, but rather reaches its target as a result of lysosomal processing (Houslay & Elliott, 1981). This work needs further investigation. The hydrophilic B subunit was not detected in the membrane by photoaffinity labelling.

The observed hydrophobicity of the H-chain of tetanus toxin suggests it could dissolve in the membrane. This correlates with evidence (from limited trypsinolysis in urea) for some conformational change when the toxin (which has a hydrolytic surface) binds ganglioside (P. Britton & S. van Heyningen, unpublished work).

The transmembrane action of cholera toxin probably involves a localized destabilization of the membrane due to multivalent binding of ganglioside G_{M1} . The B subunits would not enter the membrane, but a very high local concentration of A subunits would be induced. A very small proportion of the A1 peptides could then interact with polar molecules in the membrane to exert their effect on the inside of the cell.

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