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Analysis of the molecular basis of insecticidal specificity of *Bacillus thuringiensis* crystal δ-endotoxin

Muhammad Z. HAIDER and David J. ELLAR*
Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

The mechanism of action and receptor binding of a dual-specificity *Bacillus thuringiensis* var. *aizawai* IC1 δ-endotoxin was studied using insect cell culture. The native protoxin was labelled with ^{125}I, proteolytically activated and the affinity of the resulting preparations for insect cell-membrane proteins was studied by blotting. The active preparations obtained by various treatments had characteristic specificity associated with unique polypeptides, and showed affinity for different membrane proteins. The lepidopteran-specific preparation (trypsin-treated protoxin containing 58 and 55 kDa polypeptides) bound to two membrane proteins in the lepidopteran cells but none in the dipteran cells. The dipteran-specific preparation (protoxin treated sequentially with trypsin and *Aedes aegypti* gut proteases, containing a 53 kDa polypeptide) bound to a 90 kDa membrane protein in the dipteran (*A. aegypti*) cells but bound to none in the lepidopteran cells or *Drosophila melanogaster* cells. The toxicity of trypsin-activated δ-endotoxin was completely inhibited by preincubation with D-glucose, suggesting a role for this carbohydrate in toxin–receptor interaction. The toxicity was also decreased by osmotic protectants to an extent proportional to their viscometric radius. These results support a proposal that initial interaction of toxin with a unique receptor determines the specificity of the toxin, following which cell death occurs by a mechanism of colloid osmotic lysis.

INTRODUCTION

Bacillus thuringiensis strains produce crystalline, phase-bright cytoplasmic inclusions (δ-endotoxins) during sporulation that contain one or more insecticidal polypeptides (Somerville, 1978; Bulla *et al.*, 1980; Thomas & Ellar, 1983; Knowles *et al.*, 1986). The δ-endotoxins from different serotypes vary considerably in their specificity for different insect groups (Dulmage, 1981). This may be the result of quantitative differences in δ-endotoxin production between individual strains. However in certain cases the distinct host range of a given δ-endotoxin is accompanied by a characteristic pattern of polypeptides in the native crystal, suggesting that in these cases specificity may reside in unique proteins (Yamamoto & McLaughlin, 1981).

An additional explanation for the specificity of these δ-endotoxins centres on the possible differences in the biochemistry of the larval mid-gut between various insect groups. The need for high pH and gut proteases to activate the protoxins following ingestion, raises the possibility that variations in pH and gut protease spectrum in different insect groups may generate toxins of varying potency and specificity. This possibility has recently been investigated (Haider *et al.*, 1986) using δ-endotoxin from var. *colmeri* (now redesignated as var. *aizawai* IC1; Haider *et al.*, 1987). Specificity in this case was found to be the result of differential proteolysis of the protoxin by host gut proteases. Thus when the protoxin was activated with lepidopteran gut proteases the resulting active toxin was toxic only to lepidoptera *in vitro* and *in vivo*, whereas when it was treated with dipteran proteases, it showed toxicity only to dipteran cells and larvae. This study also showed that this change in insect-specificity was accompanied by cleavage of approx. 15 amino acids from the lepidopteran-specific 55 kDa toxin by the dipteran gut proteases to yield the 53 kDa dipteran-specific form.

The work reported here describes an investigation of the cytolytic mechanism of the crystal toxin from *B. thuringiensis* var. *aizawai* IC1.

EXPERIMENTAL

Bacterial strains and growth conditions

B. thuringiensis var. *aizawai* IC1 used in these studies was obtained as strain no. HD-847 from the Bacillus Genetic Stock Centre (Ohio State University, Columbus, OH, U.S.A.). Upon serotyping, it was found to be an *aizawai* (serotype H-7) strain and was therefore redesignated as var. *aizawai* IC1 (Haider *et al.*, 1987). Growth and sporulation conditions were as described for *Bacillus megaterium* KM (Stewart *et al.*, 1981).

Isolation and activation of δ-endotoxin crystals

The method used for crystal δ-endotoxin purification was that described by Thomas & Ellar (1983) for *B. thuringiensis* var. *kurstaki*. The purified δ-endotoxin was solubilized and then activated using trypsin, or trypsin and *Aedes aegypti* gut proteases, as described earlier (Haider *et al.*, 1986).

Radiolabelling of the δ-endotoxin

The purified crystal δ-endotoxin was labelled by ^{125}I using the method of Fraker & Speck (1978). Native δ-endotoxin (1.0 mg) was resuspended in 1.0 ml of phosphate-buffered saline (Oxoid) in a vial which was precoated with Iodogen (chloramine; 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril) (Pierce). The reaction was

* To whom all correspondence should be directed.

initiated by the addition of 0.75 mCi of carrier-free $Na^{125}I$ (Amersham), and incubated at 4 °C for 15 min with shaking. Following the incubation, the reaction was stopped by addition of an excess of phosphate-buffered saline. Unbound ^{125}I was removed by washing the crystals in phosphate-buffered saline at 14000 ***g***. The final pellet was resuspended in distilled water and stored at 80 °C until used.

Cell lines and cytotoxicity assay

The cell lines and their growth conditions have been described earlier (Knowles *et al.*, 1986; Haider *et al.*, 1986).

Cytotoxicity was assayed by counting the percentage of viable cells by Trypan Blue staining with or without the addition of activated toxin (Thomas & Ellar, 1983; Haider *et al.*, 1986).

Preparation of the membrane fraction from insect cells

Crude plasma membrane fraction was prepared essentially as described earlier (Knowles & Ellar, 1986) except that the final membrane pellet was dissolved directly in gel sample buffer instead of phosphate-buffered saline.

Electrophoresis and protein blotting

SDS/polyacrylamide-gel electrophoresis was carried out as described by Laemmli & Favre (1973) using an acrylamide:*NN'*-methylenebisacrylamide ratio of 100:1. Electrophoresis conditions and staining and destaining procedures have been described previously (Haider *et al.*, 1986).

Proteins separated by SDS/polyacrylamide-gel electrophoresis were transferred electrophoretically to nitrocellulose filters (Schleicher and Schuell) by the method of Towbin *et al.* (1979) using a Bio-Rad 'Transblot' apparatus. Non-specific binding sites were blocked by incubating the filter in 3% (w/v) bovine serum albumin (Sigma) in Tris-buffered saline (10 mM-Tris/HCl, pH 7.4, containing 0.9% NaCl) for 60 min. This was then incubated for 3 h with 100 μg of ^{125}I-activated toxin (9×10^7 c.p.m./mg) on a rocking platform. The nitrocellulose filter was washed six times with Tris-buffered saline, blotted dry and autoradiographed with Fuji X-ray film.

Preincubation of activated toxin with sugars and estimation of the toxin pore size

Mamestra brassicae cells (2×10^6 cells/ml) grown as described earlier were transferred to fresh TC 100 medium containing 300 nM of appropriate sugar at least 30 min prior to the addition of activated var. *aizawai* toxin (100 μg/ml) and cytotoxicity assays were conducted for the appropriate times. Viable cell counts at each time-plot were normalized by comparison with negative controls (i.e. cells with no toxin added).

The toxin pore size was estimated by a modified method of Weiner *et al* (1985) using *M. brassicae* cells (Knowles & Ellar, 1987).

RESULTS

B. thuringiensis var. *aizawai* IC1 δ-endotoxin was radiolabelled to high specific activity with ^{125}I, activated with trypsin or trypsin plus *A. aegypti* gut proteases and its affinity for membrane proteins of various insect cell lines was studied by blotting. The trypsin-activated toxin (containing 58 and 55 kDa polypeptides) specifically bound to two membrane components (approx. 120 and 68 kDa) in both the lepidopteran cell lines studied (*Choristoneura fumiferana* and *Heliothis zea*) (Fig. 1). However this trypsin-activated preparation did not bind to any of the dipteran cell-membrane proteins (not shown). In contrast to this, when the protoxin was activated by sequential treatment, first with trypsin and then with *A. aegypti* gut proteases, the resulting preparation (containing only the 53 kDa polypeptide) bound only to one membrane protein of molecular mass 90 kDa in the *Aedes albopictus* cells and to none of the proteins in lepidopteran (not shown) or *Drosophila melanogaster* cells (Fig. 1*b*, lane 3).

To test the possibility that these putative endotoxin receptors may be glycoconjugates, *M. brassicae* cells were preincubated with sugars before addition of toxin. A range of sugars. i.e. D-glucose, L-glucose, D-mannose, methyl D-glucoside (Fig. 2), ribose, arabinose, mannitol, lactose, sucrose, raffinose (Fig. 3), L-fucose, D-galactose, D-(+)-glucosamine, *N*-acetylgalactosamine and *N*-acetylglucosamine (not shown), were tested for their effect on the toxicity of trypsin-activated var. *aizawai* IC1 δ-endotoxin. Only D-glucose and raffinose were found to completely neutralize the toxicity of activated toxin while lactose and sucrose gave only partial neutralization. Further confirmation that the protective effect of D-

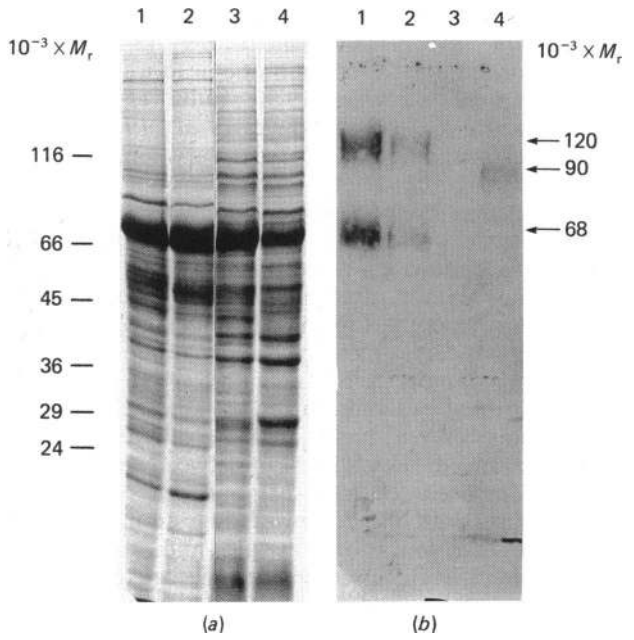

Fig. 1. Identification of the insect cell-membrane receptors for *B. thuringiensis* var. *aizawai* IC1 δ-endotoxin

(*a*) SDS/10% polyacrylamide gel (Coomassie Blue stained) of membrane proteins (100 μg) from different cell lines. Lane 1, *C. fumiferana* CF1; lane 2, *H. zea*; lane 3, *D. melanogaster*; lane 4, *A. albopictus*. (*b*) An autoradiogram of trypsin-activated [^{125}I]endotoxin incubated with the nitrocellulose blot of the same gel (lanes 1 and 2) and [^{125}I]δ-endotoxin activated first with trypsin and then with *A. aegypti* gut proteases (lanes 3 and 4). For details of the blotting procedure see the Experimental section. The lines indicate positions of M_r standards and the arrows indicate the membrane receptor proteins.

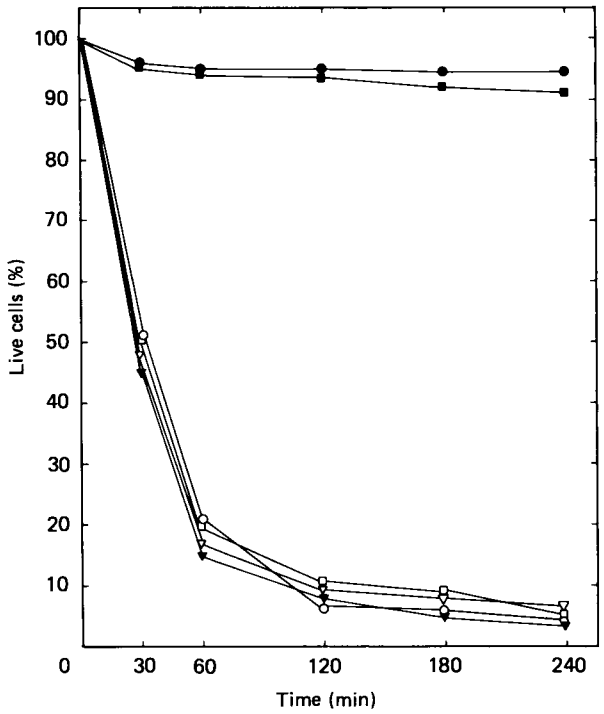

Fig. 2. Inhibition of toxicity with D-glucose

Trypsin-activated *B. thuringiensis* var. *aizawai* IC1 δ-endotoxin (100 μg) was preincubated with 300 mM-sugar for 60 min and applied to *M. brassicae* cells (1.5×10^6). The cell viability was measured by Trypan Blue staining after each time interval. (●), Control; (■), D-glucose; (○), L-glucose; (□), mannose; (▽), methyl D-glucoside; (▼), trypsin-activated var. *aizawai* IC1 δ-endotoxin.

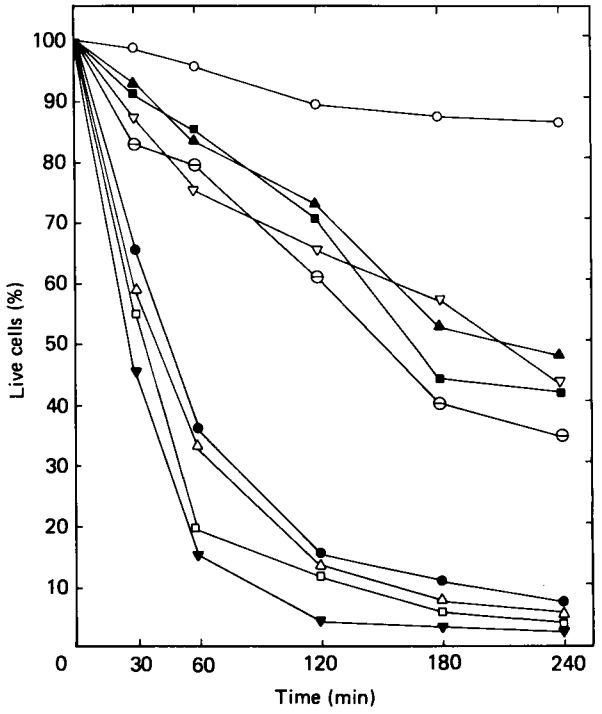

Fig. 3. Effect of osmotic protectants on toxicity

M. brassicae cells (2×10^6) were resuspended in TC 100 medium containing 300 mM-sugar for 30 min and then 100 μg/ml of trypsin-activated toxin was added to each test sample. Cell viability for different samples was measured by Trypan Blue staining. The curves show cell viability in the presence of different sugars: (○), raffinose; (▲), galactoarabinose; (■), lactulose; (▽), lactose; (⊖), sucrose; (●), mannitol; (△), ribose; (□), arabinose; (▼), trypsin-activated var. *aizawai* δ-endotoxin.

glucose was specific, was obtained from reciprocal experiments in which the activated toxin was pre-mixed with concanavalin A before addition to the cells and the effects were measured by cytotoxicity assays. A significant protective effect was observed (Fig. 4) although it was less than that observed with D-glucose (80% protection in contrast to 99% in the case of D-glucose). The percentage protection was calculated by measuring the average of the viable cells (preincubated with sugar) at each time-point in the toxicity assay and comparing them with the corresponding controls. No protective effect was observed with a variety of other lectins, e.g. soybean agglutinin, peanut agglutinin and wheat-germ agglutinin (results not shown). The disaccharides sucrose and lactose contain terminal glucose as part of the molecule which could have been the cause of the observed protection. This was further studied by using the disaccharides lactulose (4-*O*-β-D-galactopyranosyl-D-fructose; Sigma) and 3-*O*-β-D-galactopyranosyl-D-arabinose in the protection experiments. The results of these experiments (Fig. 3) show that these two sugars protect as well as lactose and sucrose indicating that protection with sucrose and lactose is not due to the presence of the glucopyranosyl residue.

The observation that among the variety of monosaccharides at equimolar concentrations that were tested, only D-glucose protected the cells against the toxin, strongly suggests that this carbohydrate is interfering specifically with toxin–receptor binding. Previous experiments with a range of *B. thuringiensis* δ-endotoxins (Ellar *et al.*, 1986; Knowles & Ellar, 1987) led us to propose that these toxins kill cells by a colloid osmotic lysis mechanism. Toxin binding to membrane receptors leads to the creation of a membrane leakage pore that allows equilibration of ions between the cell and the exterior. The accompanying net influx of water into the cell will result in cell swelling and eventual lysis. One prediction of this theory is that external molecules with viscometric radii too large to allow them to enter the toxin-induced leakage pore will act as osmotic protectants by maintaining a high external osmotic pressure and preventing water influx. This phenomenon of non-specific osmotic protection forms the basis of a method for measuring the dimensions of the toxin-induced leakage pore (Weiner *et al.*, 1985). We have applied this method to the *B. thuringiensis* var. *aizawai* IC1 toxin. *M. brassicae* cells were incubated with sugars of increasing viscometric radii. Portions of a trypsin-activated preparation were then added to each incubation and the extent of toxicity was subsequently measured. As shown in Fig. 3, arabinose, ribose and mannitol had no appreciable effect on the toxicity, sucrose and lactose showed some protection and raffinose completely protected the cells against lysis. These results were then used to calculate the time required for 50% lysis in the presence of each sugar (t_{50}), which was in turn used to obtain the toxin pore size (Weiner *et al.*, 1985). Fig. 5 indicates that the estimated pore size for the var. *aizawai* IC1 toxin is of the order of

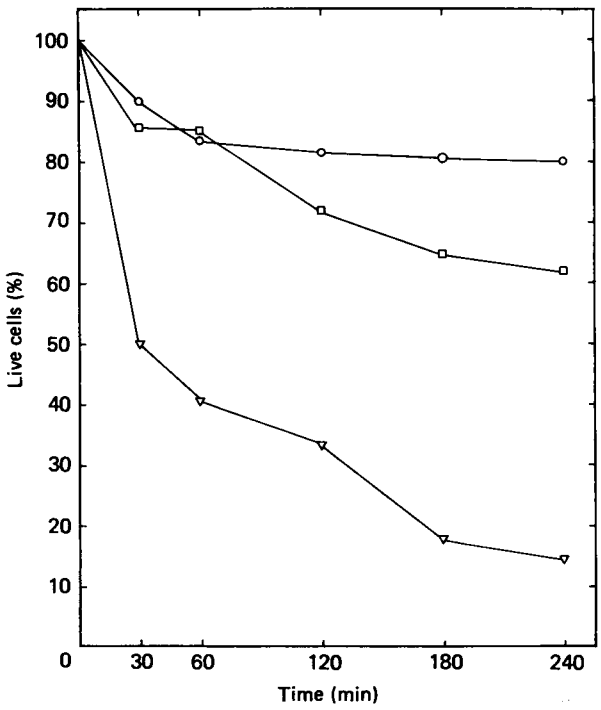

Fig. 4. Effect of concanavalin A on toxicity

Trypsin-activated *B. thuringiensis* var. *aizawai* IC1 δ-endotoxin (100 μg) was premixed with 10 μg of concanavalin A/ml and applied to 2×10^6 *M. brassicae* cells. The cell viability was recorded following Trypan Blue staining. (○), Control; (□), trypsin-activated var. *aizawai* toxin mixed with concanavalin A; (▽), trypsin activated var. *aizawai* δ-endotoxin.

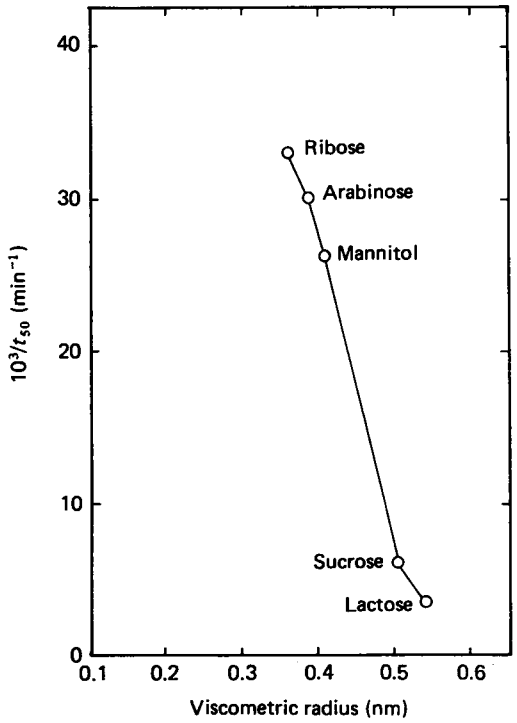

Fig. 5. Pore size estimation

M. brassicae cells were resuspended in TC 100 medium containing 300 mM-sugar. The time for 50% cytolysis (t_{50}) after addition of 100 μg of *B. thuringiensis* var. *aizawai* IC1 δ-endotoxin/ml was calculated from a time-course of Trypan Blue uptake. The reciprocal of t_{50} was plotted against the viscometric radius of the solute. Pore radius can be estimated from the point where t_{50} approaches zero.

0.6 nm. From previous measurements of the dimensions of the pore/hole induced by other *B. thuringiensis* δ-endotoxins (Knowles & Ellar, 1987), the trisaccharide raffinose, and the disaccharides used in the experiments reported here, would be too large to enter the pore and therefore the protection we observe with these carbohydrates is the result of this non-specific osmotic effect rather than the specific effect shown by D-glucose.

DISCUSSION

B. thuringiensis strains exhibit a high degree of specificity in their action on insect larvae. When tested on cells *in vitro*, the activated toxins generally have a distinct profile of toxicity (Knowles *et al.*, 1986). An exception to this is the 27 kDa toxin of var. *israelensis*, which lyses all cells tested, (Thomas & Ellar, 1983). The native *B. thuringiensis* var. *aizawai* IC1 endotoxin used here consists of two protoxin polypeptides, which are processed to 58 and 55 kDa polypeptides by trypsin-activation. This preparation is lepidopteran-specific and does not affect dipteran cells or larvae (Haider *et al.*, 1986). The blotting experiments reported here suggest that this preparation has specific affinity for the 120 and 68 kDa membrane proteins in the lepidopteran (CF1 and *H. zea*) cell lines but does not bind to any of the dipteran cell-membrane proteins.

Treatment of the trypsin-activated preparation with *A. aegypti* gut proteases yields a dipteran-specific preparation in which the 55 kDa protein is processed to a 53 kDa polypeptide and the 58 kDa protein is completely degraded (Haider *et al.*, 1986). When this preparation was tested in the cell-free membrane binding studies it bound to a single, 90 kDa protein in the membrane of *A. albopictus* cells. No binding could be detected to the membranes of the lepidopteran or *Drosophila* cells, which in the earlier study were found not to be affected by this toxin.

These results indicate that the 53 kDa polypeptide, although derived from the 55 kDa protein, recognizes a different membrane receptor, confirming the earlier suggestion (Haider *et al.*, 1986) that the loss of approx. 15 amino acids results in an alternate conformation of the molecule and a change in its specificity. Knowles & Ellar (1986) have described the interaction of the lepidopteran-specific P1 toxin of var. *kurstaki* with a 146 kDa *N*-acetylgalactosamine-containing glycoprotein receptor on CF1 cells. In contrast to *kurstaki* P1 toxin, which lyses only CF1 cells, the var. *aizawai* IC1 toxin has a much broader spectrum of activity upon appropriate proteolytic activation and also exhibits differential toxicity *in vivo* and *in vitro* to the two insect groups mentioned earlier (Haider *et al.*, 1986). It is therefore significant that different polypeptides in the activated *aizawai* IC1 preparations bind to membrane receptors in lepidopteran and dipteran cells. These results suggest that var. *aizawai* IC1 toxin-specificity is determined by (1) a

particular toxin conformation, which depends upon the type of proteolytic activation received, and (2) the presence of membrane receptor(s) specific for that conformation. Carbohydrate protection experiments were used to test the possibility that the receptor for var. *aizawai* toxin may be a glycoconjugate. The marked neutralizing effect of D-glucose suggests that it may be part of the receptor(s). Further experiments using the lectin concanavalin A confirmed this specific role of glucose. An additional feature of the membrane-binding studies reported here is that the lepidopteran toxicity appears to be due to both the 58 and 55 kDa proteins in the active preparation. In their study of *B. thuringiensis* var. *aizawai* crystal after activation with *Spodoptera frugiperda* gut extract, Lecadet *et al.* (1986) found that the presence of two polypeptides (63 and 67 kDa) was necessary for full activity against *S. fugiperda in vivo*.

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