THE ROLE OF ENVIRONMENTAL PARAMETERS ON THE STABILITY OF CHOLERA TOXIN FUNCTIONAL REGIONS

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1. Introduction

Native cholera toxin is composed of three unique polypeptide chains α, β and γ [1–3]. The α and γ chains, of which there is one per molecule, are covalently linked through a single disulfide bridge, forming protomer A. The remainder of the molecule consists of five β chains [4], forming a stable complex designated protomer B. Thus, the toxin has a molecular formula $\alpha\gamma\beta_5$. Both protomer A as well as polypeptide α stimulate adenylate cyclase in intact cells and cell-free systems [5]. Although the mode of action of the toxin is poorly understood, it is known that protomer A, in the presence of a large excess of dithiothreitol, catalyzes the hydrolysis of NAD to ADP-ribose and nicotinamide [6,7], and the transfer of the ADP-ribosyl moiety to itself [8]. The role of the ADP-ribosylated A protomer, thus formed, in adenylate cyclase activation remains to be determined. The B protomer is devoid of any enzymatic activity but binds the toxin to its cell membrane receptor, the monosialoganglioside G_{M1} [9–11] and is believed to be needed for the penetration of A into the cell [3].

Exposure of the toxin to thiol reducing agents splits the molecule into two fragments, an insoluble one the α polypeptide chain and one left in the supernatant as a complex of the two chains β and γ [2]. The study of the chemical and functional properties of the two fragments is complicated by the self-association of the α chain after reduction. Protomer A also is unusually insoluble in aqueous media. At present the only way of circumventig this problem has been to add high excesses of urea or to expose the toxin molecule to acid pH so that insolubility in the usual buffers can be avoided. On denaturation, however, the binding domains holding together the toxin molecule fall apart and the constituent polypeptide chains can be resolved [1,12,13]. Therefore all the properties, referred above, have been described for protomer A or the single polypeptide chains isolated and kept in solution under denaturing conditions.

This communication describes a study of the environmental parameters affecting the aggregation transition of the α chain and conditions for optimum stability of reduced cholera toxin in the absence of denaturing agents.

Abbreviations: G_{M1}, galactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosylglucosylceramide

2. Materials and methods

Toxin was isolated and assayed as in [14]. Care was taken to use a protein homogeneous with respect to size and charge. For reduction aliquots of toxin solution (1 mg/ml) in 0.02 M Tris–HCl buffer, pH 8.2, 0.01 M dithiothreitol, 0.002 M EDTA were incubated at 20°C for 1 h. The reduced samples were immediately exposed to the stabilizing agents under investigation. Samples were analyzed by analytical polyacrylamide gel electrophoresis with and without SDS, performed according to [14]. After staining with Coomassie blue R 250, the protein bands were located and quantitated by densitometric scanning with a mod. 240 Gilford spectrophotometer equipped with a mod. 2410 scanning unit. Results are expressed as % of the soluble α chain relative to experiments in

which the incubations with the stabilizing agent were omitted.

Difference spectral measurements were carried out, in a Varian Cary 17D recording spectrophotometer, to monitor the conformational changes of cholera toxin after exposure to the stabilizing agents, following the method in [15].

All other chemicals and methods were as in [14].

3. Results and discussion

The reduction of cholera toxin resulted in the formation of two fragments (fig.1). Molecular weight determinations with the method [16] and analyses by acrylamide gel electrophoresis with SDS revealed that the slower migrating fragment was comprised of the β and γ chains attached to form the $\gamma\beta_5$ complex (app. mol. wt 63 000), while the faster migrating fragment was identical to the α chain (app. mol. wt 20 000). The complex $\gamma\beta_5$ maintains the ability (fig.1d) to form insoluble complexes with ganglioside G_{M1} [2,17]. Thus reduction splits cholera toxin into two functional regions: the $\gamma\beta_5$ complex responsible for recognition of the cell surface receptors and the α chain responsible for the activation of adenylate cyclase.

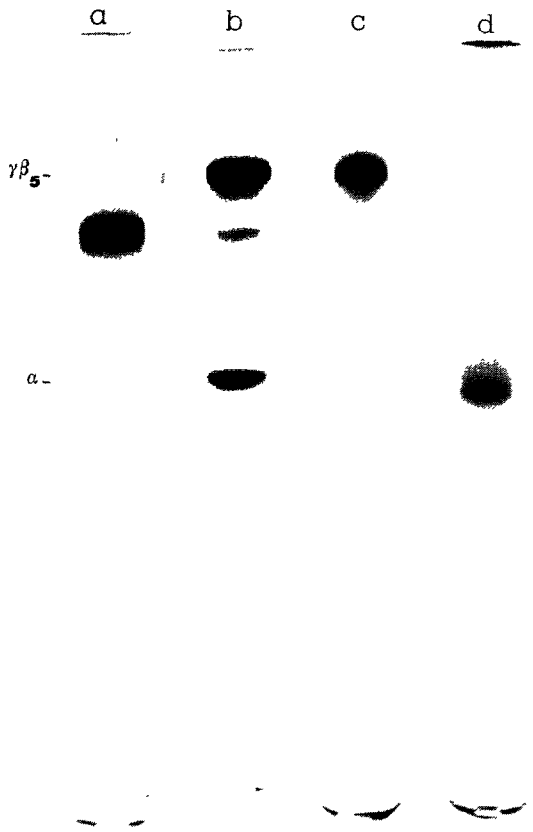

Fig.1. Polyacrylamide disc gel electrophoresis of native (a) and reduced cholera toxin (b); (c) a sample of cholera toxin at a final 2.5 mg protein/ml was reduced as in section 2 and centrifuged in order to remove the aggregated material; (d) cholera toxin was incubated in 20% (v/v) ethylene glycol with G_{M1} (0.5 mg ganglioside/mg toxin) for 10 min at 37°C, before adding dithiothreitol. Protein, 20 μg, was applied/gel.

Further studies on the properties of the functional regions are based on optimum stability of the reduced protein over long periods of time. Using standard conditions of reduction, it was found that the α chain suffers concentration-dependent aggregation. The reduced toxin shows evidence of precipitation above 1 mg/ml protein. As shown by polyacrylamide electrophoresis the high molecular weight material formed was aggregated α chain (fig.1c).

The disorganizing effect of increasing temperature is shown in fig.2. The α chain is rapidly precipitated

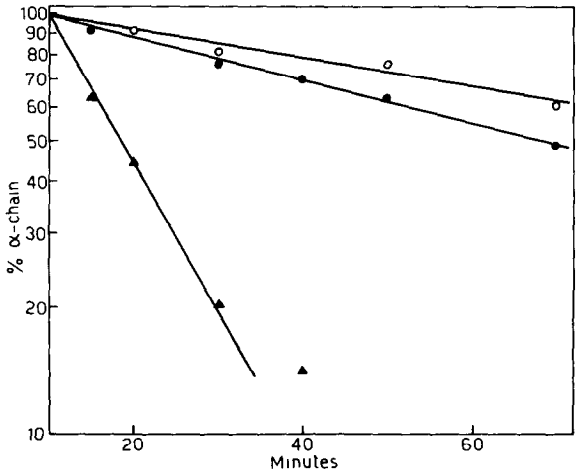

Fig.2. Thermal stability of reduced cholera toxin. Reduced cholera toxin (0.4 mg/ml) was incubated at 25°C and 37°C, with and without 20% (v/v) ethylene glycol, in a final 0.5 ml At the times indicated aliquots were quickly cooled in ice and centrifuged to remove some aggregated material. The supernatant was analyzed by polyacrylamide disc gel electrophoresis. After staining, the protein bands were quantitated by densitometric scanning. (o——o), 25°C; (▲——▲), 37°C; (●——●), 20% (v/v) ethylene glycol at 37°C.

at 37°C, where it has a $t_{1/2}$ of 17 min; it is much more stable at 25°C, where it has a $t_{1/2}$ of 90 min.

The effect of pH on the aggregation of the α chain could not be quantitated because of a broadening of the protein bands upon pH changes. At neutral pH the toxin slowly dissociates into protomers A and B [17]. At alkaline pH, deamidation of asparagine and/or glutamine residues generates microheterogeneous forms of cholera toxin of different electrophoretic mobilities [14]. However, effects in the range pH 8.0–9.5 are apt to be minor compared to the changes observed with neutral salts. Raising the ionic strength of the reduced protein solution increases the α chain aggregation. Ions, such as HPO_4^-, which are not particularly effective in dissociating non-covalently linked aggregates of proteins [18], are instead effective destabilizers of the α chain. Incubation of the reduced toxin with 0.1 M potassium phosphate buffer at pH 8.2 and 30°C caused the rapid aggregation of the α chain. More than 30% was lost within 10 min. Similar results were obtained with sodium chloride. Salts might be expected to influence the α chain stability primarly by weakening attractive charge–charge interactions between the two functional regions of the toxin. Such non-specific effects on electrostatic interactions would facilitate the α chain dissociation from the $\gamma\beta_5$ complex and its subsequent precipitation.

NAD, for which cholera toxin has a specific affinity [7,8] is not able to prevent the structural changes eventually leading to denaturation. In the presence of 2 mM NAD, the time-dependent course of precipitation of the α chain is identical at 25°C and 37°C to the one reported in fig.2 for cholera toxin alone.

Stability of reduced cholera toxin and of the α chain over long periods of time (several months) is rendered possible by adding agents such as dimethylsulfoxide, glycerol or ethylene glycol. Ethylene glycol prevents the aggregation of the α chain and protects the protein against the disorganizing effects of temperature (fig.2), ionic strength and protein concentration. This nonaqueous solvent does not alter the conformation of the protein in the concentration range investigated, as shown by the ultraviolet difference spectra (fig.3). By lowering water activity, the nonaqueous solvents tend to stabilize the intramolecular hydrogen bonds and to enhance inter- and intramolecular electrostatic interactions [19]. This new

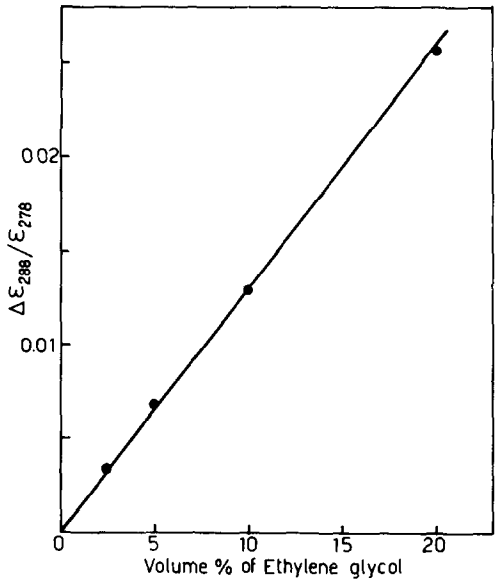

Fig.3. The effect of ethylene glycol concentration on the difference spectra of cholera toxin. The difference spectra were obtained with toxin (0.4 mg/ml) in 0.1 M Tris-HCl buffer, pH 8.2, at 25°C and the indicated amounts of ethylene glycol according to the method in [15].

structural information supports the view that, aside from the disulfide bridge, the other non-covalent binding domains holding together the two functional regions obtained from reduced cholera toxin are probably of electrostatic nature.

The reported conditions of stability of reduced cholera toxin are very promising for the study of the chemical and physiological properties of the interacting functional regions of cholera toxin, as well as for their separation. Moreover, the stability of the reduced protein gives a new tool for studies on the mechanism of action of cholera toxin since several results [7,8,20] have been obtained under conditions in which the formation of α chain precipitates is most pronounced.

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