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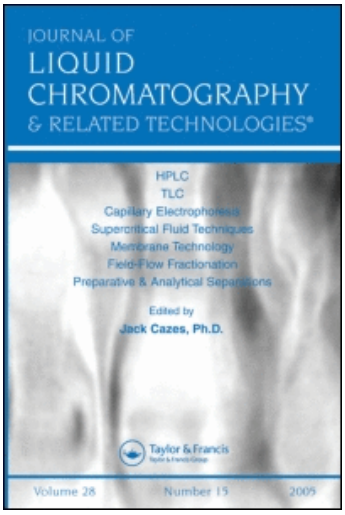

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V. E. Klyushnichenko[a]; O. N. Smirnov[b]
[a] Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Moscow, Russia [b] Research Institute of Biotechnology, Moscow, Russia

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ANALYSIS OF CONFORMATIONAL CHANGES OF CHOLERA TOXIN BY SIZE EXCLUSION, HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

V. E. Klyushnichenko,[1] O. N. Smirnov[2]

[1]Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry
Moscow, Russia

[2]Research Institute of Biotechnology
Moscow, Russia

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ABSTRACT

Cholera toxin (CT) consists of the A-subunit (ACT) and B-subunit (BCT) consisting of 5 identical beta-peptides. The dynamics of denaturation and subsequent renaturation of CT and BCT was investigated by SE HPLC. The denaturation was performed in the 6M urea at different temperature. The decomposition of CT to ACT and BCT and subsequent breaking of BCT to the beta-peptide is clearly seen at CT denaturation. The proteins were denatured completely and rapidly only when heating urea solution up to 50°C. The renaturation performed with the help of dialysis is more effective than that by chromatography. The quantitative correlation pentamer/ monomer of renatured BCT is higher than a fresh solved protein. Thus, a shift of the fluorescence maximum of BCTmight be explained by the presence of some denatured BCT in the solution.

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INTRODUCTION

Cholera toxin (CT) is a protein consisting of two different subunits: B-subunit (BCT) (56 kD), A-subunit (ACT) (28 kD), and bound noncovalently.[1] BCT properly consists of 5 identical beta-peptides and is responsible for CT binding to ganglioside GM1 on the surface of the cell-target membrane.[2,3] Pentamer of BCT has a cylinder form with the diameter 60-64 Å and height 40 Å, with the pore 30 Å long and changing the diameter - 11-15 Å.[4] The pentamer is stable because of the high ratio of the beta-peptide surface area, bound with the surface of neighboring beta-peptides, to the complete surface area of beta-monomer: 0.39.[5] ACT consists of two different peptides A1 - (22 kD) and A2 - (5kD) bound by a disulfide bridge.[6] The C-terminus of A2-peptide is bound to the BCT surface pore by electrostatic interactions. A strong interaction between N-terminal of A2-peptide and A1-peptide consists of Wan-der-Waals and hydrogen interaction. So, A2-peptide joins the component between BCT and A1-peptide.[4]

As known, each of the beta-peptides contains single, and A1-peptide 3 triytophane residues. Fluorescent spectras of renatured CT and BCT are practically identical (292 nm). At the bonding of CT or BCT to the carbohydrate fragment of ganglioside GM1, the fluorescent spectrum of BCT triytophiles changes.[7,8] However, in real investigations, the shift of the fluorescence maximum (up to 5 mN) of triytophane residues of BCT in the 0.05M NaCl, Tris-HCl, (pH 7,4) solution, and in liposome suspension in the same buffer was observed. Consequently, a small part of the protein in solution may be partially or completely denatured and forms beta-peptide.

To verify the above assumption, the dynamics of CT and BCT denaturation by urea at different temperatures, and the following chromatographic renaturation or dialysis, were researched by size exclusion high performance liquid chromatography (SE HPLC).

Figure 1. (right) Dynamics of BCT denaturation and renaturation under different conditions (λ=280). The BCT sample was treated properly: a) dissolved in 0.05 M Tris-HCl (pH 7.4), b) 6 M urea, 1 h, T=20°C, c) 6 M urea, 2 h, T=20°C, d) 6 M urea, 3 h, T=20°C, e) 6 M urea, 19 h, T=20°C, f) 6 M urea, 17 h, T=4°C, g) 6 M urea, 20 h, T=20°C, + 20 min 50°C, h) 6 M urea, 2 h, T=20°C, then renaturation by dialysis, i) 6 M urea, 19 h, T=20°C, then beta-peptide was isolated by SE HPLC and injected to the column in 1 h. k) 6 M urea, 19 h, T=20 °C, then beta-peptide was isolated by SE HPLC and injected to the column in 48 h.

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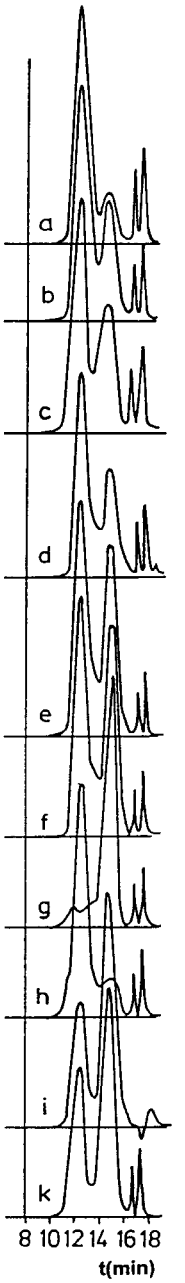

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MATERIALS AND METHODS

Chromatographic analysis was performed by a SE HPLC Protein Pack column (30 x 0.75) (Waters, Millipore, USA) in 0.1 M Na-phosphate buffer (pH 7.4); elution was performed at the rate of 0.5 mL/min. Chromatography was carried out on a Waters 510 pump with a Waters U6K injector, a Waters 490E spectrophotometer and Waters 740 integrator (Millipore, USA). For the separation we used the specimens of insulin, proinsulin, denatured proinsulin, proinsulin-S-sulphonate, fusion protein, (obtained in our laboratory)[9], bovine serum albumin, ovalbumin and lysozime. The following reagents were used: water, purified on Milli-Q equipment (Millipore, USA), sodium hydroxide, sodium phosphate, phosphoric acid, urea (Serva, Germany). Before chromatography, the eluents were filtered through nitrocellulose filters, pore diameter 0.45 μM, (Millipore, USA) and degassed for 20 min.

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Denaturation was performed in 6M urea under different temperature conditions. Renaturation was performed by dialysis and SE HPLC. At chromatographic renaturation of CT, whole proteins were separated from salts after 6M urea treatment (18 h) and then injected to the SE column for different periods of time for analysis. In the case of BCT, only beta-peptide was isolated and then analyzed, like above. BCT was renatured by dialysis against 0.05M Tris-HCl (pH 7.4) in 24h.

For picture performance, software for the IBM PC Surfer and Grapher (Golden Software Inc., USA) was used.

RESULTS AND DISCUSSION

According to SE HPLC, analysis of the initial sample of BCT, dissolved in 0.05 M Tris-HCl (pH 7.4), has two forms - pentameric and monomeric and its pentamer/monomer correlation is about 5/1 (Fig.1a). The 6M urea treatment of BCT at room temperature revealed that denaturation occurs only

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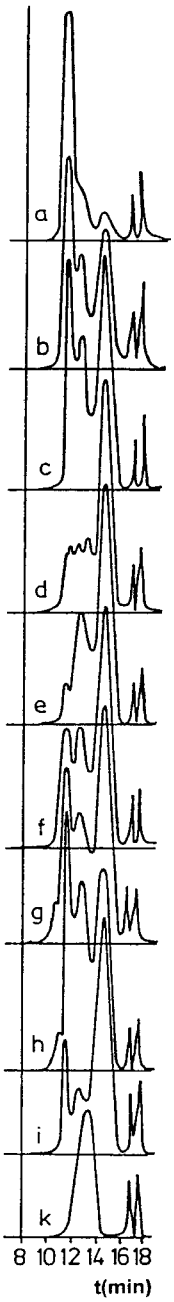
Figure 2. (right) Dynamics of CT denaturation and renaturation under different conditions(λ=280). The CT sample was treated properly: a) dissolved in 0.05 M Tris-HCl (pH 7.4), b) 6 M urea, 25 min, T=20°C, c) 6 M urea, 75 min, T=20°C, d) 6 M urea, 18 h, T=20°C, e) 6 M urea, 18 h, T=4°C, f) 6 M urea, 18 h, T=20°C, than CT sample was desalted by SE HPLC and injection to the column in 0.5h, g) 6 M urea, 18 h, T=20°C, then CT sample was desalted by SE HPLC and injected into the column in 3h, h) 6 M urea, 18 h, T=20°C, then CT sample was desalted by SE HPLC and injected into the column in 5h, i) 6 M urea, 20 min, T=50°C, k) ACT dissolved in 0.05 M Tris-HCl (pH 7.4).



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partially (about 25-30%), and that practically there is no difference in the time denaturation for 1, 2 or 3 hours (Fig.1 b-d). Even at long urea treatment (19 h), the protein is denatured less than 50% (Fig.1e). Essential differences in denaturation at room temperature and at 4° was not observed (Fig 1f). Thermotreatment with urea of the protein (50°C) during 20 min (Fig.1g), results in almost complete denaturation. It is clear, that practically the whole pentamer is transformed, and has a monomeric form.

Renaturation by dialysis, with gradual decreasing of the urea concentration, is more effective than renaturation by chromatographic desalting (85 and 30% renatured protein respectively) (Fig.1 h-k). The quantitative ratio pentamer/monomer of dialysis renatured BCT is higher than fresh dissolved protein (6/1 - 5/1 accordingly) (Fig.1 a,h). However, a small amount of high molecular weight component formation is observed here. One can suppose it is an oligomer of the beta-peptide (a small left shoulder of the first peak) (Fig.1 h). At the chromatographic purification and isolation the beta-peptide is only renatured for 35-40%, even in 2 days (Fig.1 i, k).

The initial CT sample contains some free beta-peptide (second peak) and probably BCT and ACT (right shoulder of first peak) (Fig.2 a). At the denaturation, the visible transformation of CT to BCT and ACT, and following decomposition of BCT into beta-peptide (Fig.2 b-d), is observed. The rate of CT decomposition is higher than BCT under the same conditions. As for BCT, there is no big difference in the denaturation rate at room temperature and 4°C (Fig.2 d,e). The CT denaturation occurs rapidly at temperature 50°C (Fig.2 i). It is difficult to identify the peak of ACT (Fig.2 k) at denaturing CT, because it is fused partially with BCT. However, in some cases, all components of denaturation are identified - CT, BCT, ACT, and beta-peptide (Fig.2 d). At the CT renaturation by chromatographic desalting, the increase of CT and BCT peaks (Fig.2 f) is observed in half an hour. However, even in 3-5 h, renaturation is not completed (Fig.2 g,h). Besides, high molecular weight impurities are formed (left shoulder of the first peak).

CONCLUSION

The present work, thus investigates the dynamics of denaturation at different temperature and subsequent renaturation of CT and BCT by SE HPLC and dialysis. The resultant data explains the shift of fluorescence maximum by the presence of the small amount of denatured BCT or monomeric beta-peptide, which may transfer into a pentamer after dialysis. The data may also be used for the calculation of equilibrium parameters of bonding (constant of bonding, stoichiometry) of the B-subunit of cholera toxin to GM1 ganglioside.

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