Journal of Biomedical Sciences
Research Article Peer-review, Open Access

Binding Site of *Corynebacterium Diphtheria* Toxin and Metal Ion

Walaa Shakir Mahmood [1]

Abstract

Diphtheria has been one of the most extensively studied bacterial infectious diseases of all time. These pivotal studies of the toxigenic bacterium Corynebacterius diphtheriae and its major virulence determinant (diphtheria toxin) have set the standard for other related bacterial protein toxin studies. The DtxR is a metal ion activated transcriptional regulator that is associated with the virulence of Corynebacteria diphtheriae. Structure determination indicates that there are 2 metal ion binding sites for each repressor monomer. Site directed mutagenesis indicates that binding site 2 (primary) is critical for recognition of target DNA repressor.

Using calorimetric methods, it has been shown that binding site 1 and 2 act independently of each other, and their role can be easily elucidated by conventional mutational assays. Diphtheria toxin and anthrax toxin research over the last 30 years has resulted in a comprehensive understanding of the structure function relationships of these toxins (for example, catalytic, transmbrane, and receptor binding domains) as well as identification of their Eukaryotic Cell Surface Receptors (ESRs), understanding of molecular events that lead to receptor-mediated intramuscularization of toxin into endosomal compartments, and pH-induced conformational changes necessary for the formation of pores in vesicle membranes. One of the most widely studied bacterial infectious diseases is diphtheria. In recent years, a great deal of research has been devoted to understanding in detail the molecular interactions of each of these toxins with eukaryotic cellular factors that play an important role in the rapid translocation of their catalytic domains through a pore on the trans-endosaic vesicle membrane and subsequent delivery into the cell cytochrome P450 system. These milestone studies of the toxin of toxigenic origin Corynebacterium diphtheriae and its main virulence determinant (diphtheria toxin) have set the standard for the study of related bacterial protein toxins.

Keywords: *Corynebacterium diphtheria* – respiratory vector -metal.

[1] Baghdad University –College of Science –Biotechnology department, Iraq

Introduction

Several bacterial exotoxins function by facilitating the transfer of the ADP-ribose moiety from NAD to form a covalent bond with specific target proteins found in mammalian cells. In the case of both diphtheria toxin (DT) the target protein is elongation factor 2 (EF-2). As a result of ADP-ribosylation, the factor becomes deactivated, leading to the inhibition of protein synthesis and ultimately resulting in cell death. ADP-ribosylation serves to enhance the activity of the cyclase, leading to an increase in cAMP concentrations, which in turn can produce various physiological changes depending on the type of affected cell. These treatments result in the cleavage of the toxin into two fragments: an enzymatically active A fragment (Mr, 21,167) and a B fragment (Mr, 37,195) responsible for receptor recognition and membrane translocation.

In previous studies, we extensively characterized the enzymic and ligand-binding properties of fragment A. It was discovered that fragment A possesses a single NAD-binding site with a dissociation constant (Kd) of 8 uM. This site not only catalyzes the ADP-ribosylation of EF-2 but also facilitates the slow hydrolysis of NAD to ADP-ribose, nicotinamide, and H' through the NAD glycohydrolase reaction (EC 3.2.2.5). Previous research conducted by our laboratory and others has shown that DT fragment A contains a single site for NAD, with areas for both aromatic parts of the dinucleotide. Later, we observed a linking between fragment A and NAD induced by ultraviolet light, which is likely caused by the excitation of one or both of the ligand's nitrogenous bases. Additional support for this interpretation is provided by studies involving different forms of DT. On the other hand, forms of DT with altered or blocked NAD sites, showed significantly reduced levels of labeling. Similarly, the NAD-binding site of exotoxin A is not exposed in its native form, and photochemical labeling only occurred with the activated form. Previous findings suggested that the nicotinamide moiety of NAD interacts with a tryptophan residue in the A fragment. Binding of NAD to free fragment A or the nucleotide-free form of DT leads to a significant decrease in intrinsic tryptophan fluorescence and the emergence of a new absorbance peak at 360 nm.

Both phenomena are reliant on an N-substituted nicotinamide ring and may potentially arise from a charge-transfer complex formed between the positively charged nicotinamide and an adjacent indole side chain .(1) This effective labeling of exotoxin A is not surprising, considering that the NAD-binding and enzymic properties of activated exotoxin A closely resemble those of DT fragment A. Given that the NAD dissociation constants of the other proteins tested are considerably higher than those of the two toxins, a smaller proportion of the binding sites would have been in a liganded state under the irradiation conditions employed. Further experiments are required to determine whether efficient and specific photochemical modification using nicotinamide-labeled NAD is exclusive to diphtheria toxin and exotoxin A, unique to ADP-ribosylating exotoxins, or also occurs with other classes of NAD-binding proteins.

Toxigenic strains of *Corynebacterium diphtheriae* synthesize Diphtheria toxin in a precursor form. After cleavage of its 25 amino acid signal sequence, the toxin is released into the culture medium as a single chain protein consisting of 535 amino acids. The toxin's ADP-ribosyltransferase activity is activated by proteolytic "nicking" of the α-carbon backbone at Arg193 in a 14 amino acid loop formed by a disulfide bond between Cys186 and Cys201. Under denaturing conditions, the "nicked" toxin can be separated into a 21.1 kDa N-terminal polypeptide (residues 1-193) containing the catalytic (C) domain and a 41.2 kDa C-terminal polypeptide (residues 194 to 535) carrying both the transmembrane (T) and receptor binding (R) domains. The C-domain catalyzes the NAD+-dependent ADP-ribosylation of elongation factor 2 (EF-2), inhibiting cellular protein synthesis and ultimately leading to cell death by apoptosis. Diphtheria toxin as one of the most extensively researched bacterial protein toxins, and as a prototype system, diphtheria has served as a model for the investigation of other protein toxins. diphtheria remains a leading model system, and its examination continues to offer crucial insights into the interactions between eukaryotic cell proteins and bacterial protein toxins that are vital in the process of intoxication.(2)

Fragment B contains both the T-domain and the R-domain. The R-domain, which spans residues 432-535, is responsible for binding diphtheria toxin to its cell surface receptor, known as a heparin binding epidermal growth factor precursor (hb-EGF) (3,4,5,6). Several early studies have demonstrated that diphtheria toxin requires passage through an acidic compartment to deliver its C-domain to the cytosol. These studies have shown that weak bases and amines, including ammonium salts, glutamine, and chloroquine, as well as ATP inhibitors, can block C-domain delivery. However, the use of v-ATPase inhibitors, such as Bafilomycin A-1, ultimately confirmed that the pore-forming activity of the toxin is associated with the action of the v-ATPase in the membrane of endosomal vesicles.

The crystal structure of diphtheria toxin has provided valuable insights into previous research findings and has facilitated the creation of toxin mutants at specific positions involved in translocation (7,8). To gain a better understanding of how diphtheria toxin interacts with cell membranes, researchers have employed various methods, such as enzymatic cleavage assays using either full-length toxin or fragment B in cell membranes or artificial bilayers (9). These studies have consistently shown the insertion of transmembrane helices 8 and 9. However, discrepancies arise when interpreting the cleavage products that include the C-domain and whether they represent intermediates in the translocation process However, it was not realized by (10,11) provided direct experimental evidence that cellular factors are essential for catalytic domain delivery to the cytosol and proposed a hypothesis proposing a direct involvement of cellular factors in facilitating delivery.

In this context, the requirement for receptor-mediated endocytosis and the finding that clathrin, dynamin and (v) ATPase inhibitors all block intoxication by diphtheria toxin all suggest that entry into the C-domain action process related to cellular factors that are essential but indirectly involved in (12,13,14) The diphtheria toxin repressor DtxR is a metal ion-activated transcriptional regulator associated with *Corynebacterium diphtheriae* virulence. Structural determination showed the presence of two metal ion binding sites per repressor monomer, and site-directed mutagenesis showed that binding site 2 (primary) was essential for recognition of the target DNA repressor; The role of binding site 1 (secondary) was demonstrated. remain. Unclear (14).Our results clearly demonstrate that binding site 1 (auxiliary site) is first occupied during metal ion activation and plays an important role in stabilizing the repressor. The metal ion-regulated repressor that controls transcription of diphtheria toxin repressor (DtxR) belongs to a family of virulence genes in pathogenic bacteria.

Crystallographic studies of DtxR have shown that the active form of the repressor is a homodimer composed of two domains (1).The N-terminal domain contains a helix-turn-helix motif involved in DNA binding and two binding sites for metal ions (2).Although the function of the C-terminal domain of is not completely understood. It is unlikely that it plays a significant role in activation., binds DNA with the same affinity as wild-type (WT) (3).Additionally, some members of the DtxR family essentially lack this domain (4). In addition to iron ions, DtxR is activated in vitro by other divalent transition metal ions with the following activation order: Fe2 Ni2 Co2 Mn2 (8, 9).Initial binding experiments showed that DtxR has a high affinity metal ion binding site (10, 11).

The researchers concluded that the ionic binding of metal to DtxR is cooperative based on the shape of 's binding curve (11).Although reconstitution experiments did not allow determination of relative binding affinities, reveals that binding site 1 (minor) has a higher affinity for the metal ion than binding site 2 (major).I made it .These results led the authors to conclude that there is some cooperativity in the metal ion binding between the sites and/or that protein stability is increased by the occupancy of two metal binding sites. proposed (8).NMR studies showed that binding of metal ions leads to ordering of the N-terminal domain with minimal changes in secondary structure (12).Recently, a range of relative binding affinities were measured colorimetrically and an activation mechanism was proposed . This step is followed by (further) coordination to binding site 1 to form the binding interface and complete the ordering of the N-terminal domain (3).This mechanism is in contrast to some of the experimental evidence in the literature. Its crystal structure shows metal ion coordination only at binding site 2 (primary).Metal ion coordination at this site is the only prerequisite for activation Because of this

apparent discrepancy between structural and biochemical data, binding of metal ions to the repressor in solution is necessary to clarify the exact number of binding sites required for activation and the order in which they occur. It became essential to study more thoroughly. However, the actual concentration of free iron is unknown, as are the redox states and corresponding values of *Corynebacterium diphtheriae.*

Therefore, to investigate the effect of metal ions on the oligomerization state of the protein, we measured the folding-unfolding transition of the system. Because the heat-induced folding/unfolding profile of oligomeric proteins depends on the total protein concentration, the oligomerization state of proteins can be tested by performing DSC experiments at different protein concentrations (15). In the studied concentration range, the transition temperature of metal-free DtxR (C102D) is independent of protein concentration, indicating that the protein behaves like a monomer. Identical experiments performed at pH 4.5 showed no folding-unfolding transition, suggesting that DtxR unfolds at low pH. When a protein sample is incubated in 5 mM NiCl2, aggregation occurs before the transition temperature (see figure).Four).Lower cation or protein concentrations did not reduce the extent of aggregation.

This result suggests that fundamentally different folding and unfolding behavior occurs in the presence of metal ions. One possibility is that the addition of metal ions may result in intra- or intermolecular interactions between the C-terminal and N-terminal domains. Structural data indicate that side chains from two C-terminal residues of adjacent crystallographic monomers can contribute to the stabilization of binding site 1 (auxiliary site) of DtxR and its homolog IdeR. This observation led researchers to believe that this interaction could also be established intramolecularly (7).

The problem of aggregation makes it difficult to achieve 's overall goal of determining the transition temperature of a system in the presence of metal ions and determining whether incubation with metal ions causes dimer formation. Although we were unable to do so, we were able to observe dramatic changes in the metal ions. The behavior of is consistent with such structural changes. Because the occupancy of metal ions in the crystal structures of DtxR (H79A) and DtxR (H79A,C102D) is inconsistent, we investigated the thermal stability of the systems under the same conditions used for crystallization and investigated the thermal stability of the systems under these conditions.

We checked whether stability was maintained. This could explain the absence of metal ion observed in binding site 1 (auxiliary site) were crystallized at a concentration of 1.4 M (NH4)2SO4. Under such non-physiological conditions, the stability of in DtxR can be severely compromised, and as a result, our crystal structure is likely to be induced by the crystallization environment rather than by intrinsic interactions in solution. It suggests that. Structure of DtxR with no metal ion bound to any of the binding sites. In solution, the folding-unfolding profile in 1.4 M (NH4)2SO4 shows a 5.1 °C increase in the melting temperature, and the repressor in solution by adjusting the by varying the salt concentration. This suggests that the stability of can be controlled. even for ions that have not been shown to specifically bind to repressors. NMR experiments of DtxR in solution showed that binding of metal ions produces a more ordered protein (16).

However, it is assumed that the metal ion first binds to the binding site 1 (auxiliary site) and that the structure of the repressor is obtained even in the absence of the metal ion.(17), the binding of metal ions to site 1 (auxiliary site) appears to be replaced by an increase in the salt concentration of , leading to stabilization of the protein structure. Therefore, we propose that the role of site 1 binding is to stabilize the structure. A practical model. The combined use of calorimetric and crystallographic techniques presented in this study provides a deeper understanding of the role of each metal ion binding site at the molecular level and suggests that DtxR may play a role during metal ion activation. We now have a clearer picture of the order in which various events occur. The activation mechanism can be summarized as follows. At low metal ion concentrations, DtxR exists essentially as a monomer.

As the concentration of metal ions increases, DtxR binds to metal ions in an order determined by binding affinity, with binding site 1 (auxiliary site) occupied first, stabilizing the monomer. Under the conditions of our experiments, there are no significant structural changes in the N-terminal region of the repressor

associated with binding of the metal ion in site 1 (minor).Once binding site 1 (minor) is occupied, metal ion can bind to binding site 2 (major) if metal ion concentration is high enough. At this stage, small structural changes occur in the N-terminal region. The first six residues of the N-terminal helix unwind, allowing water-mediated interactions between the carbonyl oxygen of Leu-4 and the metal ion in binding site 2 (primary) of .When a repressor is activated, dimerization occurs and the repressor is able to interact with its operator DNA.

Diphtheria toxin (DTx) is produced by *Corynebacterium diphtheriae*. Fragment A catalyzes ADP-ribosylation of elongation factor 2, which is an essential protein component of ribosomal protein synthesis. The modified form with a stretch factor of 2 is inactive. Protein synthesis stops and the cell dies. Fragment A is an active inhibitor of cell-free protein synthesis, but fragment B is required to enter intact cells. Fragment B is the binding recognition subunit. The B receptor has been identified as a glycoprotein in several cell types (18).However, the toxin has been shown to bind to protein-free membranes under low pH conditions (19), and functional insertion of A has been demonstrated in liposome target (20) found that native DTx forms anion-selective channels under acidic conditions and concluded that these pores are too small (5 Å) for A to pass through the membrane. Although the channel size calculated in has been questioned, to our knowledge no further pore size determinations have been performed using native DTx. Because direct information about the nature of toxin-membrane interactions is important for elucidating entry routes, we used photoreactive glycolipid probes (21, 22) to detect the two domains of the toxin. We determined which of the permeates the membrane bilayer. These experiments were performed using simple biological and artificial targets and both cleaved and non-cleaved forms of DTx. Considering the previous model for the involvement of lysosomes in the toxin entry process, the effect of low pH on toxin entry was also monitored (23, 24,25).

We were able to delineate the size and structure of the resulting channels. The photolabeling results obtained with DTx are similar to those for ricin toxin (26) because the A and B domains of these two potent protein synthesis inhibitors penetrate the membrane bilayer.. Therefore, the B domain of ricin and DTx does not simply bind to and remain on the surface of membrane, as has been found with cholera toxin (27, 28, 29, 30).Instead, they penetrate the membrane bilayer. The obvious difference between DTx and ricin toxin is that the former penetrates membranes better at low pH (pH 4) and the latter penetrates membranes better at high pH (pH 8) (unpublished observations).The data are consistent with toxicity studies (31, 32)..This indicates that pH affects the toxin rather than the target membrane itself. The pH-dependent differences suggest that the two toxins may use different routes to enter cells: DTx escapes from the lysosomal compartment via endocytosis, and lysine They get in through a more direct route. Although DTx cleavage dramatically increases its ability to form pores and is a prerequisite for toxicity, data obtained with both DTx and cholera toxin indicate that toxin cleavage is not required for penetration.

Range of experimental conditions. Regarding DTx, the data directly show that both A domain and B domain are involved in the entry process. The permeability data also show that DTx forms pores with a diameter of 24 Å, probably due to the dimeric structure due to aggregation. Prior to exposing the toxin to low pH, it promotes toxin binding and associated increased penetration. Preliminary observations suggest that incubating at low temperatures has the same effect. Furthermore, at low pH, there appears to be little delay between binding and permeation (unpublished data).Low pH also probably promotes pore formation in the A and B domains. Complete toxins are very inefficient at pore formation. This data is consistent with a cytosolic entry pathway involving escape from the lysosomal compartment where acid conditions can enhance functional binding. (32)

Conclusion

The combination of calorimetric and crystallographic methods presented in theis study has increased our understanding of the role of each metal ion site at the molecular level and a better understanding of the sequence in which various events occur during metal ion activation. DtxR. The activation mechanism can be summarized as follows: In the absence of metal ions, DtxR becomes a monomer. As the metal ion

concentration increases, DtxR binds to the metal ions in an order determined by affinity, initially stabilizing the monomer Under our experimental conditions, there are no significant structural changes in the N-terminal region of the repressor involved in metal ion binding. If they are empty, metal ions can bind to binding site 2 (primary) and the concentration of metal ions is high. Meanwhile, small structural changes occur in the N-terminal region. Unfolding the first six residues of the N-terminal tail results in a water-mediated interaction between the carbon oxygen of Leu-4 and metal ion 2 (first). When the repressor is activated, dimerization occurs and the repressor can interact with regulatory DNA.

References

1. Daniel Gillet, Julien Barbier, in The Comprehensive Sourcebook of Bacterial Protein Toxins (Fourth Edition), 2015:in International Encyclopedia of Public Health (Second Edition), 2017
2. The Genus Corynebacterium☆K. Bernard, in Reference Module in Biomedical Sciences, 2016Diphtheria Toxin
3. Immunotoxin Therapy for Brain TumorsV. Chandramohan, ... D.D. Bigner, in Translational Immunotherapy of Brain Tumors, 2017Diphtheria Toxin
4. Regulation of Bacterial Gene Expression by MetalsDavid J. Westenberg, Mary Lou Guerinot, in Advances in Genetics, 1997a DtxR, a functional homolog of Fur?
5. MembranesI. Iacovache, ... F.G. van der Goot, in Comprehensive Biophysics, 2012, 5.9.3.4.2 Diphtheria toxin
6. Respiratory Bacterial VaccinesLindsay K. Nicholson, Edward N. Janoff, in Mucosal Immunology (Fourth Edition), 2015Virulence Factors
7. A.A. ZasadaHistory, Science and Methods., in Encyclopedia of Food Safety, 2014, The Diphtheria Toxin
8. Plotkin, S.A.; Orenstein, W.A.; Offit, P.A. Plotkin's Vaccines, 7th ed.; Elsevier: Philadelphia, PA, USA, 2017; pp. 262–263.
9. Genus Corynebacterium. Available online: https://www.bacterio.net/genus/corynebacterium (accessed on 17 August 2022.
10. Sharma, N.C.; Efstratiou, A.; Mokrousov, I.; Mutreja, A.; Das, B.; Ramamurthy, T. Diphtheria. Nat. Rev. Dis. Primers 2019, 5, 81.
11. Sangal, V.; Hoskisson, P.A. Evolution, epidemiology and diversity of Corynebacterium diphtheriae: New perspectives on an old foe. Infect. Genet. Evol. 2016, 43, 364–370.
12. Berger, A.; Dangel, A.; Peters, M.; Mühldorfer, K.; Braune, S.; Eisenberg, T.; Szentiks, C.A.; Rau, J.; Konrad, R.; Hörmansdorfer, S.; et al. Tox-positive Corynebacterium ulcerans in hedgehogs, Germany. Emerg. Microbes Infect. 2019, 8, 211–217.
13. Fu, M.; Su, H.; Su, Z.; Yin, Z.; Jin, J.; Wang, L.; Zhang, Q.; Xu, X. Transcriptome analysis of Corynebacterium pseudotuberculosis-infected spleen of dairy goats. Microb. Pathog. 2020, 147, 104370.
14. Brodzik, K.; Krysztopa-Grzybowska, K.; Polak, M.; Lach, J.; Strapagiel, D.; Zasada, A.A. Analysis of the Amino Acid Sequence Variation of the 67–72p Protein and the Structural Pili Proteins of Corynebacterium diphtheriae for their Suitability as Potential Vaccine Antigens. Pol. J. Microbiol. 2019, 68, 233–246.
15. Canário Viana, M.V.; Profeta, R.; Cerqueira, J.C.; Wattam, A.R.; Barh, D.; Silva, A.; Azevedo, V. Evidence of episodic positive selection in Corynebacterium diphtheriae complex of species and its implementations in identification of drug and vaccine targets. PeerJ 2022, 10, e12662.

16. Dazas, M.; Badell, E.; Carmi-Leroy, A.; Criscuolo, A.; Brisse, S. Taxonomic status of Corynebacterium diphtheriae biovar Belfanti and proposal of Corynebacterium belfantii sp. nov. Int. J. Syst. Evol. Microbiol. 2018, 68, 3826–3831.
17. 369rène PitardThérèse EMalliavin Structural Biology and Molecular Modeling to Analyze the Entry of Bacterial Toxins and Virulence Factors into Host CellsJune 201911(6):
18. Diphtheria. World health Organisation (WHO), 2015.
19. de l'OMS – août 2017 Diphtheria vaccine: WHO position paper – August 2017. Vaccin antidiphtérique: Note de synthèse. Wkly Epidemiol Rec. 2017;92(31):417-435.
20. Jie Bai et al Diphtheria toxin-mediated transposon-driven poly (A)-trapping efficiently disrupts transcriptionally silent genes in embryonic stem cells. Genesis (New York, N.Y. : 2000), 58(9), e23386-e23386 (2020-07-10.
21. xenograftsVedud Purde et al Intein-mediated cytoplasmic reconstitution of a split toxin enables selective cell ablation in mixed populations and tumor .Proceedings of the National Academy of Sciences of the United States of America, 117(36), 22090-22100 (2020-08-26)
22. International Journal of Molecular Sciences AB Toxins as High-Affinity Ligands for Cell Targeting in Cancer TherapyJuly 2023International Journal of Molecular Sciences 24(13):11227
23. Parveen 1 , William R Bishai 1 , John R Murphy Corynebacterium diphtheriae: Diphtheria Toxin, the tox Operon, and Its Regulation by Fe2+ Activation of apo-DtxRSadiya 12019 Jul;7(4):10.1128/microbiolspec.GPP3-0063-2019.
24. Discovery of natural bispecific antibodies: Is psoriasis induced by a toxigenic Corynebacterium simulans and maintained by CIDAMPs as autoantigens?January 202433(1):e15014Jens-Michael Schröder
25. | Metz, B., et al. 2020.Identification of Formaldehyde-Induced Modifications in Diphtheria Toxin. | . J Pharm Sci. 109: 543-557. PMID: 31678246
26. Arnold, AE., et al. 2020. Attenuated diphtheria toxin mediates siRNA delivery. | . Sci Adv. 6: PMID: 32917630
27. Rodnin, MV., et al. 2020Structure of the Diphtheria Toxin at Acidic pH: Implications for the Conformational Switching of the Translocation Domain. . Toxins (Basel). 12: PMID: 33171806
28. Sugiman-Marangos, SN., et al. 2020Exploiting the diphtheria toxin internalization receptor enhances delivery of proteins to lysosomes for enzyme replacement therapy. | Sci Adv. 6: PMID: 33310843
29. | Ladokhin, AS., et al. 2021Conformational switching, refolding and membrane insertion of the diphtheria toxin translocation domain. | Methods Enzymol. 649: 341-370. PMID: 33712192
30. Sugiman-Marangos, SN., et al. 2022. Structures of distant diphtheria toxin homologs reveal functional determinants of an evolutionarily conserved toxin scaffold. | Commun Biol. 5: 375. PMID: 35440624
31. Hashemi Yeganeh, H., et al. 2022. A New Combination: Anti Glypican-3 scFv and Diphtheria Toxin with the Best Flexible Linker. | Protein J. 41: 527-542. PMID: 36001255
32. J. Alejandro D'Aquino, Jaclyn Tetenbaum-Novatt, Andre White Fred Berkovitch, and Dagmar Ringe 3, 2005 Mechanism of metal ion activation of the diphtheria toxin repressor DtxR.