**Vibrio cholerae** hemolysin: The β-trefoil domain is required for folding to the native conformation

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**ABSTRACT**

*Vibrio cholerae* cytolysin/hemolysin (VCC) is a 65 kDa β-pore-forming toxin causing lysis and death of eukaryotic cells. Apart from the core cytolysin domain, VCC has two lectin domains with β-trefoil and β-prism folds. The β-prism domain binds to cell surface carbohydrate receptors; the role of the β-trefoil domain is unknown. Here, we show that the pro-VCC mutant without the β-trefoil domain formed aggregates highly susceptible to proteolysis, suggesting lack of a properly folded compact structure. The VCC variants with Trp532Ala or Trp534Ala mutation in the β-trefoil domain formed hemolytically inactive, protease-resistant, ring-shaped SDS-labile oligomers with diameters of ~19 nm. The Trp mutation induced a dramatic change in the global conformation of VCC, as indicated by: (a) the change in surface polarity from hydrophobic to hydrophilic; (b) movement of core Trp residues to the protein-water interface; and (c) decrease in reactivity to the anti-VCC antibody by ~100-fold. In fact, the mutant VCC had little similarity to the wild toxin. However, the association constant for the carbohydrate-dependent interaction mediated by the β-prism domain decreased marginally from ~3 × 10^11 to ~5 × 10^10 M⁻¹. We interpret the observations by proposing: (a) the β-trefoil domain is critical to the folding of the cytolysin domain to its active conformation; (b) the β-prism domain is an autonomous folding unit.

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1. Introduction

*Vibrio cholerae* cytolysin/hemolysin VCC; [1-3] is a β-pore-forming toxin β-PFT; [4-6] that causes colloid osmotic hemolysis of mammalian erythrocytes and apoptosis of epithelial and immune cells by forming 14-stranded heptameric channels of diameter ~1 nm in the plasma membrane bilayer [7-9]. VCC, expressed by virtually all *V. cholerae* El Tor O1 and non-O1 strains [10], is released into the growth medium as the inactive precursor, 79 kDa pro-VCC. The mature 65 kDa VCC is generated by removal of the 15 kDa N-terminus Pro-domain by endogenous or exogenous proteases at a site in the 15-residue cleavage motif of the 29-residue flexible linker joining the domain to the N-terminus of the cytolysin domain [11]. The exact site of the cleavage depends on the specificity of the protease. VCC is considered a major accessory virulence factor of *V. cholerae* [10,12].

The analysis of the amino acid sequence [2] and X-ray crystallography [13] revealed that the ~725-residue ~79 kDa pro-VCC is arranged into four structural domains (Fig. 1): the ~125-residue N-terminus Pro-domain that shows sequence homology to the Hsp90 family of heat-shock proteins, the ~250 amino acid cytolysin domain that bears similarity to the structure of the cytolysin domains of other β-PFTs, notably *Staphylococcus aureus* α-toxin [14] and is central to the structure and function of VCC as a β-PFT [4-6], a ~116 amino acid domain related to the carbohydrate-binding domain B of the plant toxin ricin with β-trefoil fold [15] and a ~140-residue domain related to the sugar-binding domain of the plant lectin jacalin with β-prism fold [16]. With the exception of the β-trefoil lectin domain, which has not been studied so far but from indirect evidences is thought to be functionally redundant, all domains have been assigned specific biochemical roles. The Pro-domain locks the assembly of the toxin monomer to the β-barrel by interacting with the cytolysin domain (Fig. 1) and prevents premature oligomerization [11,13]. The cytolysin domain mediates oligomerization and membrane penetration [2,13]. The C-terminus β-prism lectin domain mediates targeting of the toxin to cell surface β1-galactosyl-terminated glycoconjugates [17-20]. Although the domains appear to be discrete structural and functional units (Fig. 1), they profoundly affect the global properties of pro-VCC and VCC by mechanisms not related to their assigned roles. The pro-domain plays a chaperone-like role in regulating folding of the nascent polypeptide to its native conformation and, in absence of the domain, the mature VCC is expressed as functionally inactive aggregates highly...
susceptible to proteolytic degradation [21]. Similarly, the cryo-electron microscopy [22] and X-ray crystallography [23] of the membrane-inserted β-barrel VCC heptamer revealed that there is considerable realignment of the β-prism domain relative to the cytolysin domain during oligomerization. We showed by solution study that the domain promotes VCC assembly to the β-barrel heptamer by making a significant contribution to the entropy of oligomerization [24]. Although deletion of the β-prism domain has no effect on the global conformation of the toxin monomer, the presence of the domain augments the hemolytic activity of VCC by ~1000-fold [17–20,24]. The observations illustrate how discrete domains communicate with each other to increase the functional efficiency of a multidomain protein.

No function has been assigned to the β-trefoil domain, which sits on the top of the cytolysin domain covering an area of 298 Å at the interface (Fig. 1). Intriguingly, while the β-prism domain with a definite role in pore formation is missing in most of the β-PFTs related to VCC, the β-trefoil domain without an apparent biological function is a recurrent feature of all these toxins [13,25]. In this communication, we have produced VCC variants that either lack the β-trefoil domain or have the domain modified by mutation of Trp532 or Trp534 by Ala. We show that the domain makes a critical contribution to the folding of the hemolytically active conformation of VCC.

2. Materials and methods

2.1. Preparation of wild-type and mutant toxins: homogeneity and hemolytic activity

The wild-type toxin as well as the mutants were prepared by cloning Pro-VCC [11,13] into the pET32a+ expression vector (Novagen) and transformed into Shuffle™ T7 competent Escherichia coli cells (New England Biolabs). The hexahistidine-tagged pro-VCC was isolated by chromatography of the cell lysate on Ni2+–agarose column. The pro-domain including the histidine tag was removed by trypsinization at an enzyme: substrate ratio of 1:500 at room temperature for 20 min. The mature toxin was purified by hydrophobic interaction chromatography on phenyl-Sepharose CL-4B [24].

The internal β-trefoil lectin domain encoded by the 366 bp long nucleotide sequence was deleted by PCR based mutagenesis. First, a forward (C) and a reverse (B) mutagenic primer encompassing a region of 20 complementary bases not including the sequence to be deleted were designed. Next, a forward VCC primer (A) from the 5′ end of the VCC gene containing the BamHI restriction site and a reverse primer (D) from the 3′ containing the XhoI restriction site were designed. Two initial PCRs were performed using primer pair A and B together and pair C and D together which gave amplified product of 1377 bp (PCR 1) and 408 bp (PCR 2) respectively. For fusion of the amplified fragments, 1 μl product from PCR 1 and 3 μl product from PCR 2 were taken together as templates and the PCR was done for 10 cycles without primer. This step produced a nucleotide sequence of 1785 bp joining the two amplified fragments devoid of the β-trefoil gene. The fragment was amplified by using primers A and D and was cloned in pGEM-T Easy Vector System (Promega, USA) using TA cloning method and transformed into JM109 cells. The plasmid was purified from positive colonies screened by PCR, purified and the sequence confirmed by DNA sequencing. Following digestion by BamHI and XhoI, the product was ligated with pET32a+ vector digested with the same pair of restriction enzymes. The ligated plasmid was transformed into T7 competent cell and the integrity of the pro-VCC β-Trefoil sequence was confirmed by DNA sequencing.

Site-directed mutagenesis was carried out by a PCR-based method by using pET32a+ plasmid according to the protocols of QuickChange® Site-Directed Mutagenesis Kit (Stratagene). Primers for the mutation of the two sites in the β-trefoil domain of VCC, viz. Trp532Ala and Trp534Ala were obtained from Sigma Aldrich and the sequences were designed according to the instruction of the manufacturer. The mutation was confirmed by automated DNA sequencing (ABI PRISM 3100). The protein concentration was quantified by a modified Lowry protocol [26] relative to bovine serum albumin as standard. The homogeneity of the protein preparation was monitored by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% gel [27]. The hemolytic activity was assayed against a 1% suspension of washed rabbit erythrocytes in phosphate-buffered saline (PBS), pH 7.0 following incubation at 25 °C for 1 h.

2.2. Monitoring of protease susceptibility

VCC and its mutants were examined for their intrinsic resistance to proteases arising from possessing a folded compact structure by incubating the proteins with trypsin and chymotrypsin at a high enzyme: substrate weight ratio of 1:50 for 15 min at 25 °C. The reactions were terminated by adding an equal volume of 5% trichloroacetic acid. Following incubation at 4 °C for 1 h, the protein precipitates were washed thrice with 9 volumes of cold acetone, dissolved in boiling 10 mM sodium phosphate buffer, pH 7.8 containing 1% SDS and 5% 2-mercaptoethanol and subjected to SDS-PAGE [27].

2.3. Triton X-114 partitioning

To see if the mutant toxins possessed a hydrophobic surface similar to the wild-type VCC [28], proteins were dissolved in 2% Triton X-114 (Sigma) at 4 °C and separated into two phases by raising the temperature to 25 °C [29]. Following centrifugation at 10,000 × g for 10 min at 25 °C, equal volumes of aliquots were
Fig. 2. Effect of mutation of the β-trefoil domain on electrophoretic mobility in PAGE. About 4 μg of protein was applied to each lane. The gels were stained in Coomassie Brilliant Blue. A, SDS-PAGE in 10% gel of pro-VCC (lane 2), pro-VCC without the β-trefoil domain (lane 3) and VCC (lane 4). A mixture of the three variants of the wild toxin, viz. pro-VCC, VCC and the 50 kDa VCC without the β-prism domain was included as reference (lane 1). PAGE under non-denaturing conditions in 6% (B) and 10% (C) gel. Lane 1, VCC; lane 2, VCCW532A; lane 3, VCCW534A; lane 4, pro-VCC mutant without the β-trefoil domain. D and E, Trypsin digestion pattern of pro-VCC and pro-VCC without the β-trefoil domain by SDS-PAGE in 10% gel. Protein (5 μg) was incubated with trypsin at an enzyme: substrate weight ratio of 1:500 at 25 °C for 20 min in 50 mM Tris-HCl buffer, pH 8.0 and processed as described in the text. Lane 1, mixture of pro-VCC, VCC and the 50 kDa VCC variant without the β-prism domain; Lane 2, untreated pro-VCC (D) and pro-VCC without the β-trefoil domain (E); Lane 3, trypsin-digested pro-VCC (D) and pro-VCC without the β-trefoil domain (E).
Fig. 3. Quantification of the effects of Trp mutation of the β-trefoil domain on carbohydrate-dependent interactions of VCC with asialofetuin (A) and rabbit erythrocyte stroma (B) by ELISA. Each point represents the average of three independent readings with error bars indicating standard deviation.

2.4. Quantification of affinity for asialofetuin and rabbit erythrocyte stroma by Enzyme-linked Immunosorbent Assay (ELISA)

Asialofetuin was prepared by treatment of fetuin (Sigma) with neuraminidase (Sigma) in 50 mM sodium acetate-acetic acid buffer, pH 5.0 containing 50 mM CaCl2 [30]. Rabbit erythrocyte stroma was prepared by hypotonic lysis of washed erythrocytes with 5 mM sodium phosphate buffer, pH 8.0 at 4 °C. Wells of a 96-well polystyrene plate (Nunc) were coated by overnight incubation with 100 μl of asialofetuin or the stroma suspension (1 μg protein/well) in 50 mM sodium carbonate, pH 9.0. The free sites were blocked by 3 h incubation with 2% defatted milk powder in PBS followed by incubation with the wild or mutant toxin diluted serially in PBS [17,24]. The immobilized toxin was quantified by sequential incubation with rabbit antiserum to VCC diluted 1:500 in PBS, and anti-rabbit IgG conjugated to peroxidase (1:1000, Sigma). Each incubation step was preceded by washing wells with 200 μl of 0.05% Tween-20 in PBS thrice. VCC or its mutants bound to the immobilized toxin was quantified by color development with hydrogen peroxide and o-phenylenediamine at 492 nm. Apparent association constants were calculated by KyPlot.

2.5. Transmission electron microscopy

VCCW532A and VCCW534A in PBS were adsorbed on a glow-discharged carbon-coated copper grid, negatively stained with 2% uranyl acetate and examined under a JEOL JEM 1400 transmission electron microscope (Jeol Ltd., Tokyo, Japan) at 80 kV. Images were analyzed using DIGITAL MICROGRAPH software provided with an Ovius SC200B CCD camera (Gatan, Oxfordshire, UK).

3. Results and discussion

3.1. The β-trefoil domain was required for correct folding of pro-VCC

Unlike the C-terminus β-prism domain that can be nicked by proteolysis from the rest of the molecule, the β-trefoil domain lying in-between the cytolytin and β-prism domains [2] could not be removed from pro-VCC or VCC by proteolysis. So, we produced the pro-VCC variant without the β-trefoil domain by recombinant DNA technology (lane3, Fig. 2A). In contrast to the wild pro-VCC, which is monodisperse with a molecular weight of 79 K [11], the pro-VCC minus the β-trefoil domain formed large water-soluble aggregate(s) that failed to penetrate the 6% polyacrylamide gel in the absence of SDS (lane 4, Fig. 2B). Limited proteolysis of the wild pro-VCC produced the mature toxin with proteolysis stopping with the cleavage of the Pro-domain; however, the pro-VCC without the β-trefoil domain showed extensive fragmentation under similar conditions of proteolysis (lane 3, Fig. 2E), indicating an open structure with wide accessibility of the peptide bonds to a protease.

3.2. Modification of the β-trefoil domain by Trp mutation led to increase in effective size with inactivation of hemolytic activity

Next, we examined if a structurally altered β-trefoil domain affected the folding of pro-VCC. Because Trp has a much larger van der Waals volume (238 Å3) in comparison to that of Ala (92 Å3) and is also more hydrophobic as well as hydrophilic than Ala [31,32], it seemed likely that the replacement of either Trp532 or Trp534 by Ala would have serious spatial and energetic implications for the conformation of the domain. Accordingly, we selected the two Trp residues for point mutation. Both VCCW532A and VCCW534A appeared to be even more strongly resistant to proteolysis by trypsin and chymotrypsin than the wild toxin (Fig. 4A) indicating that they have compact structures characteristic of a properly folded protein. However, both the Trp mutants showed significantly reduced electrophoretic mobility in 6% (Fig. 2B) and 10% (Fig. 2C) polyacrylamide gels under non-denaturing conditions, indicating that they have significantly larger effective hydrodynamic volumes than the wild VCC. VCCW532A and VCCW534A showed 500- and 250-folds, respectively, less hemolytic activity than the wild-type VCC. Both the mutants showed strong hemagglutinating activity toward rabbit erythrocytes without causing hemolysis; interestingly, the behavior of the mutant toxins was reminiscent of that of the renatured wild-type toxin unfolded by urea [34] and indicated a multimeric lectin-like structure.

3.3. Trp mutation of the β-trefoil domain caused a marginal decrease in carbohydrate-dependent interaction of VCC

Because the hemolysis is a composite rather than a single event, a drastic decrease in hemolytic activity as a result of the Trp mutation could arguably arise from the inactivation of the initial interaction of the mutant toxins with the erythrocyte surface or abrogation of membrane events like self-assembly and membrane insertion [4-6]. To explore the first possibility, we quantified the binding of the toxin to immobilized asialofetuin (Fig. 3A), a β1-galactosyl-terminated glycoprotein inhibitor of VCC [17,24] and rabbit erythrocyte stroma (Fig. 3B) by ELISA. In contrast to the total inactivation of carbohydrate-binding activity caused by the
deletion of the β-prism domain or mutation of Asp617 [19,20,24], mutations in the β-trefoil domain caused a marginal decrease in the affinity of VCC for asialofetuin and the erythrocyte stroma that corresponded to a decrease in association constant by less than one order of magnitude from \( \frac{1}{C_m} = 3 \times 10^7 \) M\(^{-1}\). The data suggested that the ricin-like β-trefoil lectin domain did not significantly contribute to the lectin-like interaction of VCC with asialofetuin and stroma; secondly, Trp mutation of the β-trefoil domain had little or no impact on the β-prism domain.

3.4. Mutation of Trp532 and Trp534 to Ala Induced Dramatic Conformational Transition of VCC to a New Folded State

The solubility in water and the resistance to proteolysis suggested folded conformations for VCCW532A and VCCW534A. Because the mutants were hemolytically inactive and failed to self-assemble in lipid vesicles (data not shown) but retained carbohydrate-binding activity, they must have conformations different from that of the wild toxins. In contrast to VCC, which had a strongly hydrophobic surface [27], VCCW532A and VCCW534A...
protein core as well as on the protein-water interface [31,32]; of hydrophobicity and hydrophilicity, Trp is adapted to exist in the
fl
[33]. Because inversion of the surface polarity and spectro-
metry at the periphery of a central hollow [22,24]. The
bilayer or liposomes showed ring-like molecules of diameter
3.5. VCCW532A and VCCW534A are ring-shaped molecules

Previously, we observed that the transmission electron micro-
graph of the wild-type VCC monomer failed to reveal a distin-
guishing morphology, presumably due to the limited resolving
power of the electron microscope in comparison to the X-ray
crystallography. The VCC heptamer formed in the membrane lipid

transmission electron micrographs of aqueous suspensions of
VCCW532A (Fig. 5A) and VCCW534A (Fig. 5B) showed morpho-
logical similarities to the β-barrel VCC heptamer [22,24]. The rings
appeared to be uniform in shape; however, unlike the β-barrel
heptamer, they were SDS-labile and were significantly larger with
mean diameters of ~19 nm. Furthermore, the mutant rings tended
to stack as cylinders of varying heights in contrast to the
lateral aggregates formed by the VCC β-barrel heptamers in the
absence of detergents.

3.6. Conclusion

The present study shows that the folding of the pro-VCC
polypeptide to its so-called native conformation is critically de-
pendent on the β-trefoil domain. It appears, in retrospect, that the
hemolytically active conformation is unusually sensitive to minor
perturbation, e.g. nondenaturing concentrations of urea (~2 M)
trigger switchover to an inactive aggregate that retains carbohy-
drate-binding activity suggesting that the so-called native con-
formation of VCC might not be the most stable arrangement of the
protein [34]. It is tempting to compare the role of the β-trefoil
domain with that of the Pro-domain, which is also required for
folding of the VCC polypeptide to the β-PFT [21]. In comparison,
the β-prism lectin domain is an autonomous folding unit with no
requirement for the Pro- or the β-trefoil domain. The role of the
N-terminus Pro-domain is restricted to protein folding and does
not extend to its post-folding functioning. Since we could not
cleave off the internal β-trefoil domain, we are not sure if its role is
limited to regulating folding or extends to the post-folding func-
tioning of the toxin.

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Conflict of interest

Authors declare that they have no conflict of interest with the
content of this manuscript.
Author's contributions

A. M. designed and performed most of the experiments. All authors contributed to designing, performing and interpreting experiments. K.K.B. conceived the study, interpreted the experiments and wrote the paper. All authors agree to the content of the manuscript.

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Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.09.009.

References

[1] A. Zitzer, I. Walew, M. Palmer, S. Bhakdi, Characterization of Vibrio cholerae cytolysin as an oligomerizing pore-forming toxin, Med. Microbiol. Immunol. 184 (1995) 37–44.
[2] R. Olson, E. Gouaux, Vibrio cholerae cytolysin is composed of an α-hemolysin-like core, Protein Sci. 12 (2003) 379–383.
[3] A.K. Rai, K. Chattopadhyay, Vibrio cholerae cytolysin: structure-function mechanism of an atypical β-barrel pore-forming toxin, in: A. Chakraborty, A. Suroila (Eds.), Biochemical Roles of Eukaryotic Cell Surface Macromolecules, 842, Springer International Publishing Switzerland, 2015, Chapter 7, 109–125.
[4] A.P. Heuck, R.K. Tweten, A.E. Johnson, β-Barrel pore-forming toxins: intriguing dimorphic proteins, Biochemistry 40 (2001) 9063–9065.
[5] R.J.C. Gilbert, Pore-forming toxins, Cell. Mol. Life Sci. 59 (2002) 832–844.
[6] M.D. Peraro, F.G. van der Goot, Pore-forming toxins: ancient, but never really out of fashion, Nat. Rev. Microbiol. 14 (2016) 77–92.
[7] O.V. Krasilnikov, J.N. Murathkodaev, A. Zitzer, The mode of action of Vibrio cholerae cytolysin: the influences on both erythrocytes and planar lipid bilayers, Biochim. Biophys. Acta 1111 (1992) 7–16.
[8] H.K. Saka, C. Bidinost, C. Sola, P. Carranza, C. Collino, L. Bocco, C. Larroquet, J. Echenique, J.R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenique, J. R. Echenic...