

The Invertase Inhibitor Nt-CIF from Tobacco: A Highly Thermostable Four-helix Bundle with an Unusual N-terminal Extension

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Plant invertases are sucrolytic enzymes essential for plant metabolism and development. Enzyme activity is regulated on a posttranslational level *via* inhibitory proteins, referred to as invertase inhibitors. Ectopic expression of invertase inhibitors in crop plants has high biotechnological potential. However, little biochemical and up to now no detailed structural information is available about this class of plant regulatory proteins. Here, we present the crystal structure of the cell wall-associated invertase inhibitor Nt-CIF from tobacco at a resolution of 1.87 Å. The structural model reveals an asymmetric four-helix bundle with an uncommon N-terminal extension that appears to be critical for the structural integrity of the protein. Structure analysis of a second crystal form grown in the presence of CdCl₂ reveals two metal binding sites. Nt-CIF is highly thermostable and retains full inhibitory activity after cooling to ambient temperatures. The structure of Nt-CIF provides the first three-dimensional information source for the posttranslational regulation of plant invertases. Based on the recently discovered sequence homology between inhibitors of invertases and pectin methylesterases, our structural model is likely to represent a scaffold also used for the regulation of the latter enzymes, which do not share sequence similarity with invertases. Thus, our structural model sets the 3D-stage for the investigation of posttranslational regulation of invertases as well as pectin methylesterases.

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Introduction

Plant acid invertases catalyze the hydrolytic cleavage of the transport sugar sucrose,¹ which is the major transport form of carbohydrates in higher plants. Sucrose is exported from the source tissues (leaves) and transported *via* the phloem to the different sink tissues (roots, stem, reproductive organs and vegetative storage organs). Cells in the

target tissue may take up sucrose symplastically or apoplastically, and the sucrose can be hydrolyzed by invertases subsequently. These enzymes reside in the vacuole and the extracellular space, where invertase activity facilitates long-range carbohydrate transport by creating sucrose concentration gradients.² Sucrose and its hydrolysis products glucose and fructose provide growing tissues with energy and can serve as signals regulating gene-expression.³ Therefore, invertase activity at the wrong time and place can dramatically affect plant viability and development.⁴ Antisense repression of invertase genes distorts seedling morphology and leaf-to-root ratios⁵ and induces male sterility in tobacco.⁶ Thus, invertases are tightly regulated in order to maintain normal plant development. While control of invertase activity can occur at the level of transcription,⁷ the

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Abbreviations used: Nt-CIF, *Nicotiana tabacum* cell wall inhibitor of beta fructosidase; PME, pectin methylesterase.

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posttranslational regulation appears to be of equal importance. Hereby, activity is modulated *via* interaction with highly specific inhibitory proteins, a general strategy also found for other enzymes involved in carbohydrate metabolism.^{8,9} These invertase inhibitors have been shown to be heat-stable, non-glycosylated monomers, affecting invertase activity in a pH-dependent manner.¹⁰ The inhibition process can be modulated *in vitro* by the substrate sucrose and divalent cations in the millimolar range.^{10,11} Biotechnological relevance has been pointed out in the case of cold-induced sweetening of potato tubers. Transgenic expression of invertase inhibitors can interfere with this major food storage problem.¹²

Recent plant genome sequencing projects have identified a novel protein family formed by invertase inhibitors and the related inhibitors of pectin methylesterase (PME),¹³ the latter being involved in the control of pectin metabolism. Common features of this plant-specific family include an N-terminal signal peptide, four conserved cysteine residues involved in the formation of two disulfide bridges^{13,14} and a total size of about 18 kDa. *In vitro* assays have confirmed that particular members of the protein family either inhibit invertase or pectin methylesterase activity, but never both.¹⁴

The limited availability of pure protein components has been a major obstacle in the biochemical and structural investigation of invertase–inhibitor interaction. As a first step to characterize the inhibition mechanism in detail, we have recently expressed and crystallized a biologically active invertase inhibitor, termed Nt-CIF (*Nicotiana tabacum* cell wall inhibitor of beta fructosidase),¹⁵ previously cloned from tobacco.¹⁶ The crystal structure reported here reveals the first three-dimensional model for a member of the inhibitory protein family. In addition, we show that the recombinant protein is heat-stable and retains biological activity upon cooling to ambient temperature. Using site-directed mutagenesis along with protein deletions, we have identified major determinants of the inhibitors' structural integrity. The presented work implies relevance for the posttranslational inhibition of pectin methylesterases, since (based on sequence homology) the structures of their cognate inhibitors are likely to be similar to our Nt-CIF structure.

Results and Discussion

Nt-CIF was expressed, purified and crystallized as described.¹⁵ Briefly, overexpression as a thioredoxin A fusion protein in *Escherichia coli* Origami cells resulted in soluble protein that was purified in a three-step procedure¹⁵ and crystallized in four different crystal forms at pH values ranging from 4.6 to 9. The structure was determined by the multiple isomorphous replacement (MIR) method using the crystal form (native 1) grown in the pres-

ence of CdCl₂ (see Table 1). Using the coordinates for molecular replacement in a second crystal form (native 2) yielded a model that has been refined including data up to 1.87 Å resolution, as described in Materials and Methods (see Table 1). The description below is primarily based on this structure that comprises all 147 residues and 122 water molecules.

Overall structure: an asymmetric four-helix bundle

Nt-CIF forms an asymmetric four-helix bundle in up-down topology with a shorter C-terminal helix pair. The bundle is preceded by an N-terminal extension forming a helical hairpin along with an additional small helix (see Figure 1(a), see below). A secondary structure assignment is included in Figure 1(c). The core of the bundle is stabilized by hydrophobic interactions involving primarily non-aromatic residues and by a disulfide bridge (Cys73 and Cys114) connecting helices $\alpha 5$ and $\alpha 6$. Analysis of the surface charge distribution revealed an acidic area originating from residues in those two helices (Figure 1(b)). The significance of this is presently unclear (see below).

Four-helix bundles represent a common topological fold found in numerous proteins, either stand alone like in cytochrome *c*, or as an integral part of a larger structural entity.¹⁷ A homology search with the program DALI¹⁸ returned as top hit a mitochondrial protein kinase involved in α -ketoacid metabolism (Z-score 8.4),¹⁹ aligning 116 residues of Nt-CIF with a four-helix bundle that in this case is an integral part of the enzyme.

Unusual thermal stability of Nt-CIF

Using circular dichroism (CD) spectroscopy (see Materials and Methods), we have estimated the melting temperature of Nt-CIF to be approximately 70 °C at pH 4.0 (Figure 2(b)). After heating the protein at 95 °C for several minutes and cooling to ambient temperature, the inhibitor retained its folding state as concluded from CD spectra (Figure 2(a) and (b)). In addition, no loss in activity was observed in invertase inhibition assays (see Hothorn *et al.*,¹⁵ data not shown). These data are consistent with our structural analysis, accounting for loop regions of minimal lengths, disulfide bridge stabilization as well as tight structural integration of N and C termini. The high thermal stability of invertase inhibitors, in particular under oxidizing conditions, may be relevant for their *in vivo* function. The expression of cell wall invertase inhibitors and their target enzyme(s) is strongly regulated during plant development^{7,20,21} (Manuela Link, S. G. & Thomas Rausch, unpublished data). In particular, these inhibitors, together with their target enzymes, are assumed to form a metabolic switch responsible for the regulation of photo-assimilate transfer to the developing seeds. Therefore, their function must be assured at high

Table 1. Summary of crystallographic analysis

	Native 1	Pb(NO ₃) ₂	K ₂ Pt(CN) ₄	Native 2
<i>Data collection</i>				
Space group	P2 ₁ 2 ₁ 2 ₁			C222 ₁
Unit cell <i>a</i> , <i>b</i> , <i>c</i> (Å)	59.2, 95.6, 126.2			60.7, 106.4, 55.8
Wavelength (Å)	0.934	0.933	0.933	0.93
Resolution (Å)	2.0	2.50	2.85	1.87
Highest shell (Å)	2.15–2.0	2.60–2.50	3.0–2.85	2.0–1.87
No. unique reflections	49,059 (9428)	47,532 (5303)	31,378 (4502)	15,167 (2455)
Multiplicity	7.3 (7.1)	3.2 (3.1)	6.6 (3.8)	8.3 (5.3)
<i>I</i> / σ ^a	16.09 (7.72)	14.24 (4.43)	17.44 (4.83)	23.8 (8.92)
<i>R</i> _{sym} (%) ^a	8.1 (25.8)	6.6 (35.6)	10.2 (34.0)	5.7 (12.7)
Completeness (%)	100 (100)	99.6 (100)	99.7 (100)	97.8 (88.2)
<i>MIRAS analysis</i>				
Resolution (Å)		15–3.0	15–3.0	–
Phasing power ^b		1.17	0.71	–
Anomalous phasing power ^b		0.6	0.41	–
Number of sites		4	3	–
Figure of merit ^b	0.44			–
<i>Refinement</i>				
Resolution range (Å)	19.67–2.0			19.25–1.87
No. reflections	49,059			15,112
<i>R</i> _{work} (%) ^b	21.2			19.4
<i>R</i> _{free} (%) ^b	25.5			23.2
Number of atoms				
Protein	4388			1107
Cd ²⁺	8			–
Bis-Tris	64			–
Solvent	236			122
R.m.s. deviations				
Bond length	0.014			0.012
Angles	1.5			1.5

^a As defined in XDS.²⁹^b As defined in CNS.³⁰

ambient temperatures. Furthermore, as the cell wall compartment represents an oxidizing environment, the structural stability of invertase inhibitors must be maintained under these conditions.

An N-terminal extension and its importance for structural stability

A helical hairpin comprising residues 3–24 followed by a short helix (residues 25–31) packs approximately perpendicular to the N (α 4) and C-terminal (α 7) helices of the bundle core (Figure 1(a)), respectively. The bundle–hairpin interface is stabilized primarily through hydrophobic residues (Figure 3), suggesting that pH-variations affecting activity do not induce major conformational changes of this area. In fact, crystal structures covering crystallization conditions in the pH range from 4.6 to 9 are strikingly similar with respect to the bundle–hairpin interface (M.H., unpublished results). The C terminus of Nt-CIF apparently contributes to interface stabilization, consistent with deletions in this area rendering the protein inactive (data not shown).

While four-helix bundles are common structural scaffolds in proteins, the N-terminal extension as seen in our structure appears to be unprecedented according to the results of the program DALI.¹⁸ It is nevertheless reminiscent of a helical hairpin in

Rho protein specific guanine nucleotide dissociation inhibitor (RhoGDI), where a similar hairpin is a major contributor to GDI activity.^{22–24} In order to find out whether the additional helical feature is important for the inhibitory activity of Nt-CIF, we carried out mutagenesis studies. However, partial (Δ 1–15) and full (Δ 1–22) deletion of the helical hairpin as well as removal of the whole extension including the third small helix (Δ 1–29) (Figure 1) resulted in heavy protein precipitation after cleavage of the fusion partner, possibly by exposing the hydrophobic bundle–hairpin interface at the surface of the remaining molecule. We have probed the importance of the disulfide bridge in this segment by substituting Cys8 by Ser, resulting in soluble but highly aggregated Nt-CIF using purification schemes similar to that of the wild-type protein. This suggests a structural role of the disulfide bridge, consistent with inactivation of Nt-CIF upon DTT treatment.¹⁶ Similarly, disrupting the salt-bridge between Asp24 and Arg26 (Figure 3) by substituting the latter with Glu resulted in heavy protein precipitation during purification, supporting again a structural role of the underlying interactions. Investigating the pattern of residues conserved between PMEIs and invertase inhibitors, it appears that they are involved in intramolecular interactions suggesting a common role for structural stability.

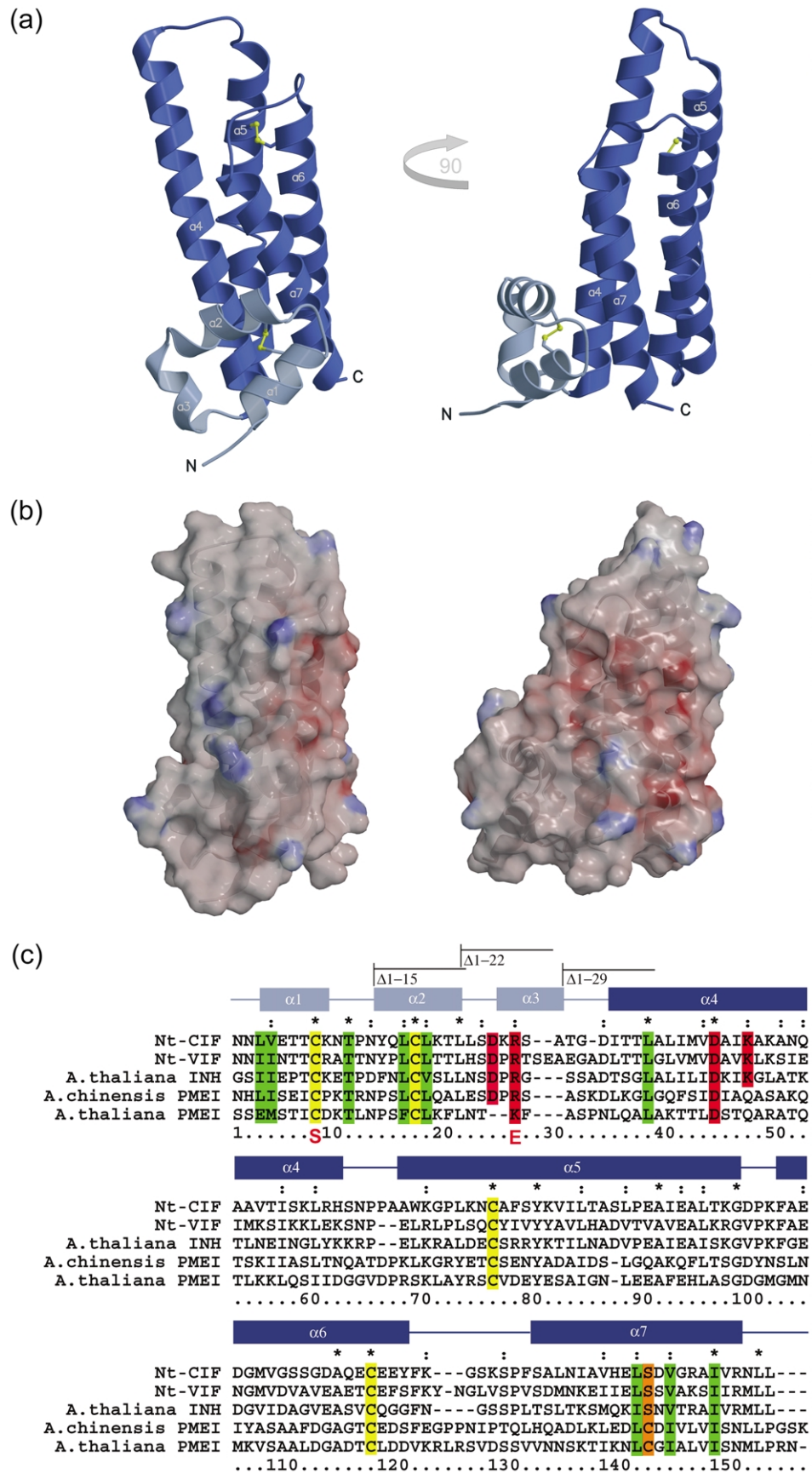


Figure 1. Structure of cell wall invertase inhibitor Nt-CIF. (a) Ribbon representation of the four-helix bundle in two orientations separated by a 90° rotation about a vertical axis, as indicated. (b) Surface view with electrostatic potential included to indicate the acidic path discussed in the text, orientations as in (a). (c) Sequence comparison of representative invertase inhibitors with secondary structure assignment according to the program DSSP³⁷ included.

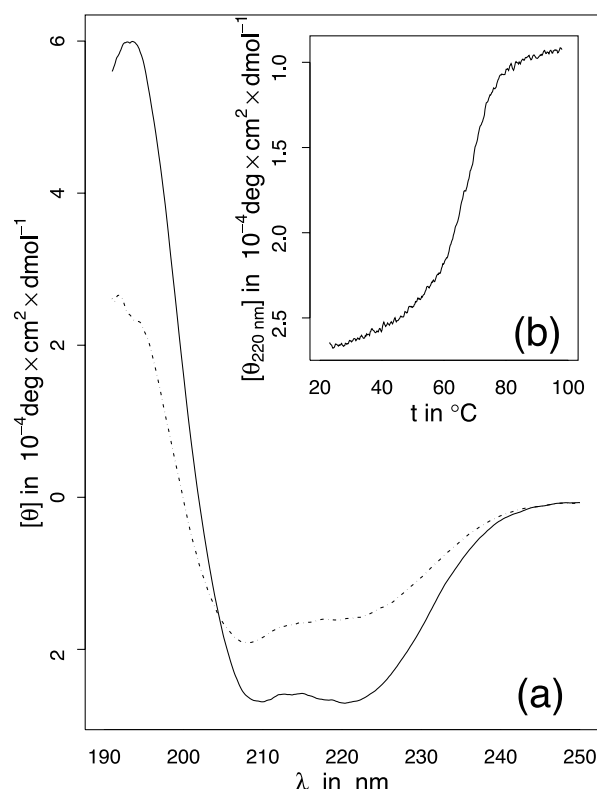


Figure 2. Circular dichroism spectra of Nt-CIF. (a) Representative far-UV spectrum at 20 °C (continuous line) and 70 °C (dash-dot line). (b) Melting spectrum of Nt-CIF at a wavelength of 220 nm and within a temperature-range of 20–98 °C. Wavelength spectra were averaged over 30 runs.

Implications for the pH dependence of inhibition

Invertase inhibition has been demonstrated to be strongly pH-dependent (Weil *et al.*,¹⁰ and literature cited therein). The tempting hypothesis that pH-variation may affect the interface between the N-terminal extension and the bundle core, is inconsistent with experimental data and the hydrophobic nature of this interface. Alternatively, the observed pH dependence of invertase inhibition might be explained as follows. In the plant cell, invertase metabolism operates in the cell wall compartment where the pH ranges between 4 and 6. Cell wall invertase activity is strongly pH-dependent with a maximum activity at about pH 4. Thus, it may be speculated that the inhibitor gets access to the binding site only in the active enzyme conformation. As the cell wall invertase is a highly basic protein, the acidic patch at the inhibitor's surface might play a role in the initial inhibitor

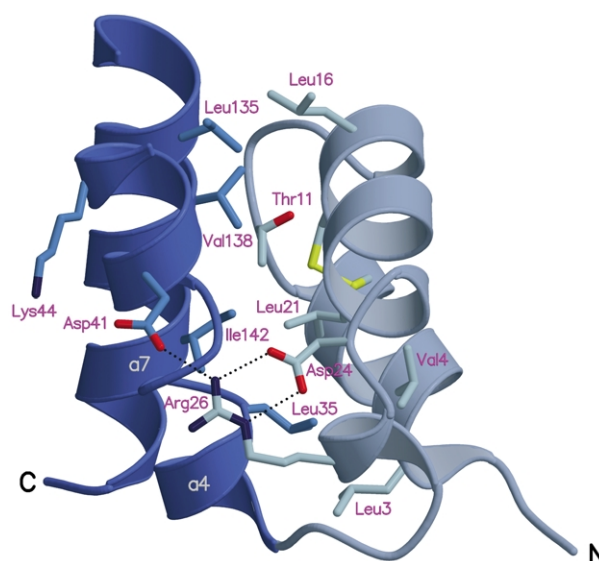


Figure 3. Close-up view of the interface buried between the N-terminal helical extension and the bundle core with selected side-chains included.

recruitment to the target enzyme, whereas other structural features such as the N-terminal hairpin might mediate the required specificity.

Does Nt-CIF bind divalent cations?

During our crystallization experiments¹⁵ we identified a crystal form obtained in the presence of CdCl₂. The structural analysis revealed two spherical density maxima per molecule present at contour levels of 18 σ in the $2F_o - F_c$ and $F_o - F_c$ map and absent from crystals grown in the absence of CdCl₂. These peaks were interpreted as cadmium ions and subsequently included in the model. Glu113 in concert with a molecule of bis-Tris buffer coordinates the first cadmium, thereby contributing to the interface with a symmetry-related molecule. In contrast, His133, Asp137 and two water molecules establish a second site located in the C-terminal helix of the bundle (Figure 4).

Since modulation of the inhibition activity by divalent cations such as Ca²⁺, Mg²⁺ and Zn²⁺ has been reported previously,¹⁰ we have investigated their influence on the inactivation of cell wall invertase by Nt-CIF. However, preincubation of the inhibitor with CdCl₂ or ZnCl₂ even in millimolar concentrations did not lead to significant changes in invertase activity (data not shown). This suggests that the effect of metal ions observed earlier¹⁰ is mediated through a more complex mechanism.

Regions and positions mutated are indicated with the corresponding mutation in red. The four cysteine residues involved in two disulfide bridges are shown in yellow, the fifth cysteine in PMEIs is highlighted in orange. Conserved residues, contributing to the bundle-hairpin interface, are indicated, dependent on their properties, in green or red.

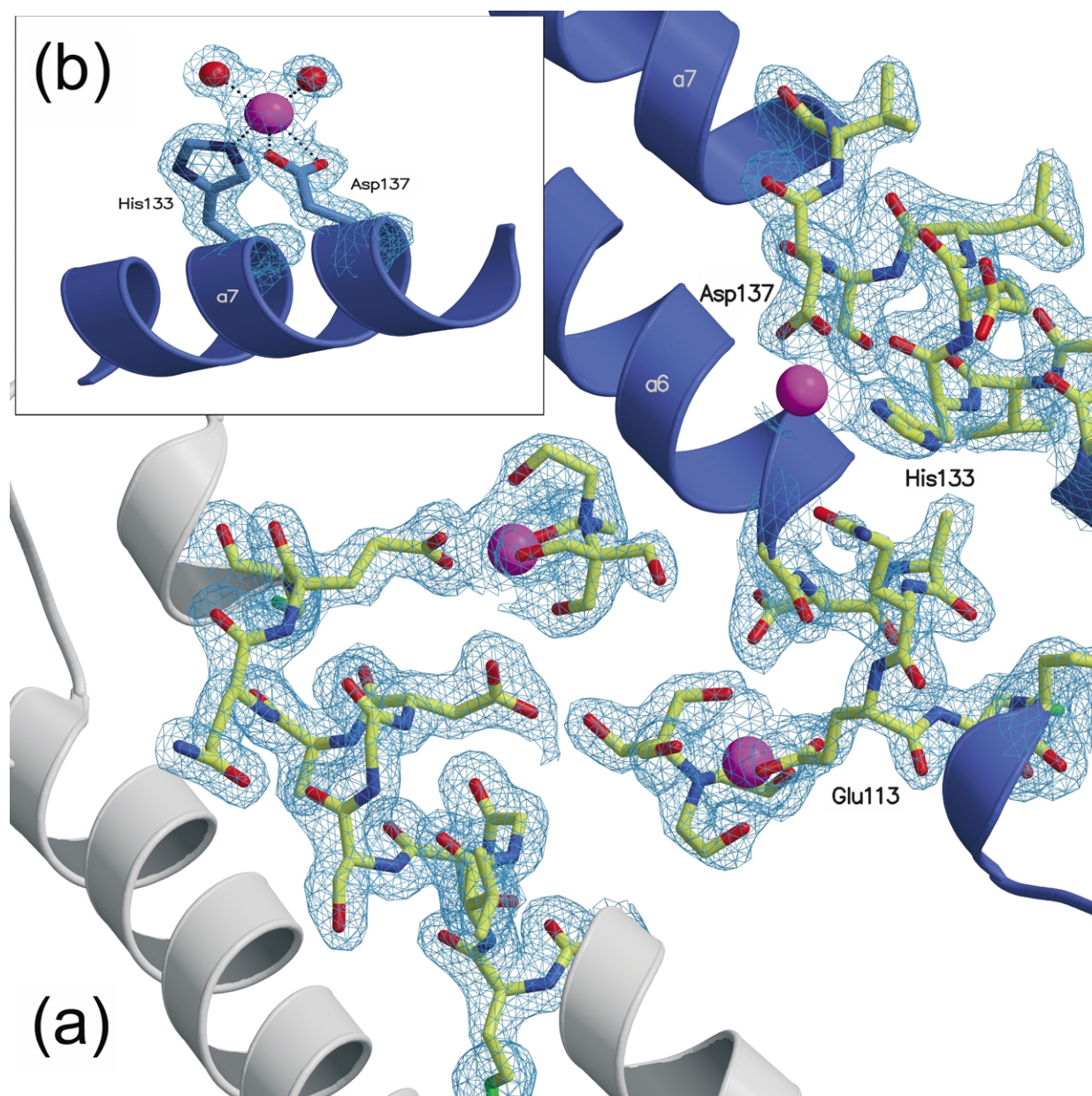


Figure 4. Metal ions in Nt-CIF. Ribbon representation of the metal binding regions with chelating residues along with bis-Tris molecules in bonds representation and including the final $2F_o - F_c$ electron density map (contoured at 1.5σ) covering the respective areas. (a) Interface between two symmetry-related molecules of Nt-CIF involving a cadmium ion (in magenta) coordinated by Glu113 along with one molecule of bis-Tris. (b) Second cadmium coordination site involving His133, Asp137 and two water molecules (in red).

Concluding Remarks

Based on secondary structure prediction and data presented,^{13,14} inhibitors of invertase and pectin methylesterase appear to be structurally very similar. The fifth conserved cysteine residue in PMEIs is replaced by serine in Nt-CIF (Figure 1(c)) pointing towards the center of the bundle. Since we have mapped other conserved residues within the protein family to be of predominant structural importance, it seems likely that specificity towards two totally unrelated enzymes is achieved by a complex pattern of surface properties, as indicated for Nt-CIF. Two

recent X-ray structures of bacterial²⁵ and plant²⁶ PME did not reveal insight into regulatory aspects. Plant invertases are highly glycosylated proteins that are found in the extracellular acidic compartments (cell wall and vacuole) whereas PMEs are exclusively targeted to the cell wall. These enzymes do not share apparent similarity in the primary sequences. However, the sequence similarity of their cognate inhibitors suggests the inhibitory power to be implemented on a similar structural scaffold. Thus, our structure offers a platform for biochemical experiments to elucidate the mechanistic details of the underlying protein–protein interactions.

The expression of invertase inhibitors during plant development is tightly regulated, suggesting that, together with their target enzymes, these inhibitors serve as a metabolic switch from a hexose_{high}/sucrose_{low} to a sucrose_{high}/hexose_{low} state. The molecular basis of this switch and the signal triggering it are to be determined. Our structural analysis in four different crystalline environments make it unlikely that pH variations induce conformational changes in Nt-CIF directly, but rather act in a more complex scheme to modulate invertase inhibitory activity. Thus, a pH-dependent change in target enzyme conformation, allowing access of the inhibitor to the binding site exclusively near the pH optimum of cell wall invertase, could explain the observed pH effects (see above).

A recent analysis of tobacco Nt-CIF-promoter:: β -glucuronidase (GUS) transformants has revealed that Nt-CIF and cell wall invertase may be co-expressed along the conducting tissues (phloem parenchyma) and in the funiculus structure, which attaches the developing seed to the mother plant (Manuela Link, S. G. & Thomas Rausch, unpublished results). In both cases, the regulation of cell wall invertase by a specific inhibitor protein may be part of a rapid posttranslational silencing of invertase activity, required for a proper orchestration of sucrose metabolism in response to endogenous and/or environmental cues. Thus, upon wounding, cell wall invertase expression is induced in the leaves along the conducting tissues, a process that may be required for the observed source–sink transition.²⁷ Here, the co-expression of the inhibitor may be required to fine-tune the spatial and temporal window of invertase activity. During seed filling, a switch from high to low cell wall invertase activity has been established for several plant species.^{20,28} This switch has been shown to be responsible for the transition from the cell division phase to the phase of storage compound accumulation. Again, the co-expressed inhibitor may be involved in fine-tuning this transition. We conclude that although the *in vivo* regulation of inhibitor–invertase complex formation has not yet been elucidated, this post-translational mechanism of enzyme regulation appears to be involved in several important developmental processes. The resolution of the first invertase inhibitor structure is another step towards a better understanding of this control mechanism.

Materials and Methods

X-ray analysis

Bacterial protein expression, purification and crystallization of the presented inhibitor from tobacco are reported elsewhere.¹⁵ Briefly, Nt-CIF was expressed as a thioredoxin A fusion protein in *E. coli* Origami cells (Novagen), and crystallized by using the hanging-drop method with 4 M sodium formate, in 0.1 M bis-Tris (pH 7) as precipitant. Heavy atom derivatives were prepared by soaking orthorhombic crystals grown in the presence

of CdCl₂ in crystallization buffer supplemented with 0.5 mM Pb(NO₃)₂ and 1 mM K₂Pt(CN)₄, respectively. Diffraction data were collected at beamline ID14-2 of the European Synchrotron Radiation Facility (ESRF), Grenoble, France. An initial native dataset (not shown) has been collected at beamline BW7-A, Deutsches Elektronen Synchrotron (DESY), Hamburg. Data processing was carried out with the program XDS.²⁹ Subsequently, isomorphous difference pattern analysis with CNS³⁰ yielded four Pb sites giving rise to an initial electron density map at 3.5 Å resolution. The contribution of three additional Pt sites from another derivative along with density modification and solvent flattening resulted in an improved map at 3.0 Å resolution comprising four molecules in the asymmetric unit. The model was built using a template bundle and completed in alternating cycles of manual model correction (O,³¹) and refinement (CNS,³⁰). The addition of cadmium ions, buffer and water molecules was carried out in the final stage of refinement against a dataset at 2.0 Å resolution (native 1) collected at ID14-2. A summary of the crystallographic analysis is given in Table 1.

The structure of the cadmium-free form (native 2) has been determined by molecular replacement using one refined Nt-CIF molecule as search model in calculations with the program CNS. The solution (starting R_{free} 42.8%) comprises one molecule in the asymmetric unit superimposing well with the model derived from the first crystal form and lacking strong electron density at the cadmium binding sites (Table 1).

Inspection of the refined models with PROCHECK³² did not reveal any unfavorable ϕ, ψ combinations. Structure visualization was done with POVSCRIPT³³ and Raster3D.³⁴

Activity assay for inhibitor function

Tobacco cell-wall invertase has been prepared from cell-suspension cultures as described.¹⁰ Inhibition assays of invertase activity were performed following the protocol of Weil & Rausch.³⁵

Circular dichroism (CD) spectroscopy

For CD spectroscopy, protein samples were diluted to 0.05 mg/ml and dialyzed against 5 mM sodium acetate (pH 4.0), 7.5 mM NaCl. The measurements were performed on a J-710 spectropolarimeter (Jasco) at a cell-path of 2 mm and at a sensitivity of 50 mdeg. Melting curves were recorded at a wavelength of 220 nm covering a temperature range between 15 °C and 99 °C. Spectra were analyzed using the statistical package R.³⁶

Site-directed mutagenesis

Site-specific mutations were introduced with the QuickChange mutagenesis kit (Stratagene) following the manufacturer's instructions and subsequently verified by DNA-sequencing. Protein expression and purification followed the protocols established for wild-type Nt-CIF.¹⁵ Finally, the purified inhibitor was analyzed by analytical size-exclusion chromatography using a Superdex 75 HR 10/30 column (Pharmacia).

Atomic coordinates

Coordinates and structure factors have been submitted

to the Protein Data Bank (<http://www.rcsb.org>) with codes 1RJ4 (native 1) and 1RJ1 (native 2).

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