The Unusual Binding Mode of Cnicin to the Antibacterial Target Enzyme MurA Revealed by X-ray Crystallography†

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We present the X-ray structure of the antibacterial target enzyme MurA in complex with its substrate UNAG and the sesquiterpene lactone cnicin, a potent inhibitor of the enzyme. The structure reveals that MurA has catalyzed the formation of a covalent adduct between cnicin and UNAG. This adduct, which can be regarded as a noncovalent suicide inhibitor, has been formed by an unusual "anti-Michael" 1,3-addition of UNAG to an α,β-unsaturated carbonyl function in cnicin.

Introduction

We recently reported that several sesquiterpene lactones (SLs[a]) from herbal origin are potent inhibitors of the bacterial enzyme MurA, an enzyme that is involved in the synthesis of murein precursors.[1] MurA is an important target enzyme for the development of novel antibacterial drugs.[2,3] It catalyzes the transfer of an enolpyruvyl unit from phosphoenolpyruvate (PEP) to UDP-*N*-acetylglucosamine (UNAG). The antibiotic fosfomycin mimicks PEP and binds covalently to Cys115 of MurA, one of the key residues in the active site of the enzyme. (cf. Figure 1) A major drawback of fosfomycin is the fact that MurA is rendered resistant toward the compound by the single-residue mutation Cys115 → Asp (numbering according to *E. coli* MurA). Various pathogenic bacteria, e.g., *M. tuberculosis*, constitutively express the Asp115 variant of MurA and are therefore innately resistant toward fosfomycin.[4]

During the catalytic cycle, the enzyme MurA experiences significant conformational changes that are evidenced by various X-ray structures of the enzyme in the liganded and unliganded state.[5] In short, the enzyme consists of two globular domains at whose interface lies the active site. Upon substrate binding, the two domains approach each other and a flexible, superficial loop containing the Cys115 residue closes the active site.[6]

The structure−activity relationships of the newly discovered MurA inhibitors as well as various biochemical assay procedures indicated that the mode of inhibition was most likely irreversible.[1] The highly active sesquiterpene lactones such as cnicin (cf. Figure 1) contain an α,β-unsaturated carbonyl function that can act as a substrate-mimicking, electrophilic agent binding to Cys115 of MurA. In agreement with this hypothesis, we found the Cys115 → Asp mutant enzyme to be resistant toward the SLs.

Results and Discussion

Structure of the MurA−Cnicin Complex. To support further drug design work on this important antibacterial target protein, we cocrystallized MurA of *E. coli* in the presence of the SL cnicin from *Cnicus benedictus* L., the most potent MurA inhibitor within this series, whose antibacterial activity has been described before.[7,8] The inhibition of MurA by either fosfomycin or cnicin depends on the presence of the substrate UNAG, therefore, this compound was included in the crystallization setup.[1,2] The structure of *E. coli* MurA in complex with fosfomycin (MurA−fos) and UNAG has been reported before at a resolution of 1.8 Å (PDB code 1UAE).[9] Using modified crystallization conditions, we were able to obtain crystals of the MurA−cnicin complex (MurA-cni). Remarkably, the crystal growth was considerably faster for MurA−fos (2 days) than for MurA−cni (10 days).

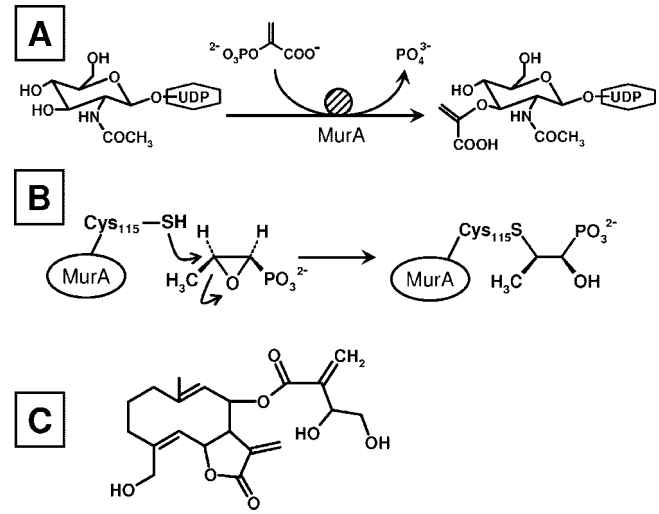

Figure 1. (A) MurA catalyzes the formation of enolpyruvyl-UNAG from UNAG and PEP. (B) The antibiotic fosfomycin covalently binds to Cys115 in the active site of MurA. (C) Structure of cnicin, a sesquiterpene lactone from *Cnicus benedictus L.*

In the 2.0 Å MurA−cni structure presented here, the enzyme adopts the "closed" conformation with the UNAG molecule localized at the expected binding site. Two unexpected observations were made (see Figure 2): First, the macrocyclic part of the cnicin molecule is not present. Second, and contrary to our expectations, there is no covalent adduct between MurA-Cys115 and the Michael-acceptor side chain of cnicin. Instead, the cnicin side chain is bound to UNAG in a way that mimicks the tetrahedral intermediate

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[a] Abbreviations: SL, sesquiterpene lactone; UNAG, Uridine-diphospho-*N*-acetylglucosamine; PEP, phosphoenolpyruvate; cni, cnicin; THI, tetrahedral intermediate; DTT, dithiothreitol.

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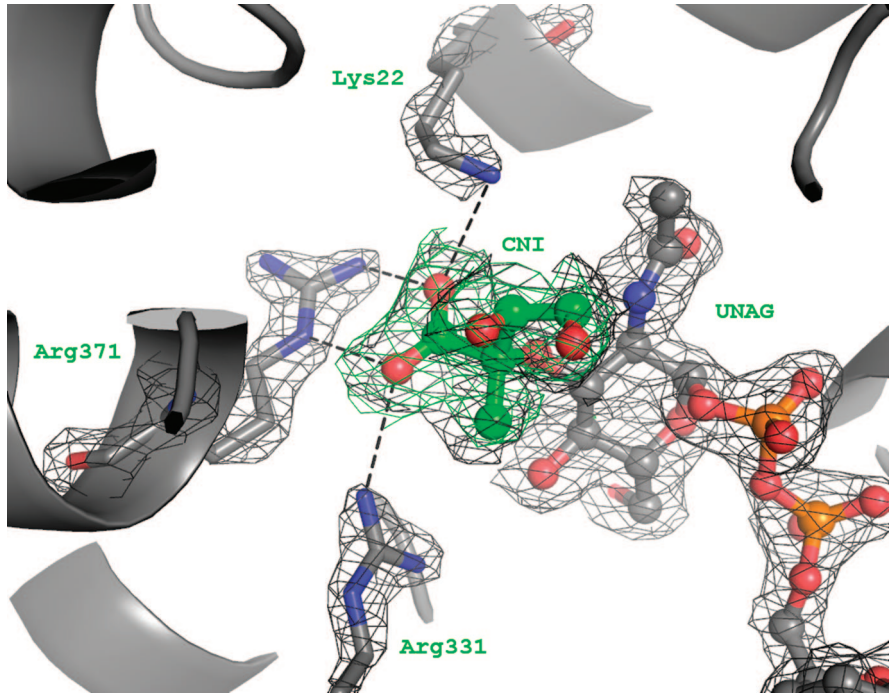

Figure 2. Structure of the MurA active site with the modified UNAG molecule. Two σA-weighted omit maps are superimposed with the final model. In gray, the 2mFo-DFc (1.5 σ-cutoff level), and in green the mFo-DFc (1.5 σ-cutoff level) electron density map around the UNAG molecule as calculated within the program REFMAC5. These maps represent omit maps because they display the electron density at an early stage of the refinement where the UNAG modification was not included in the structural model.

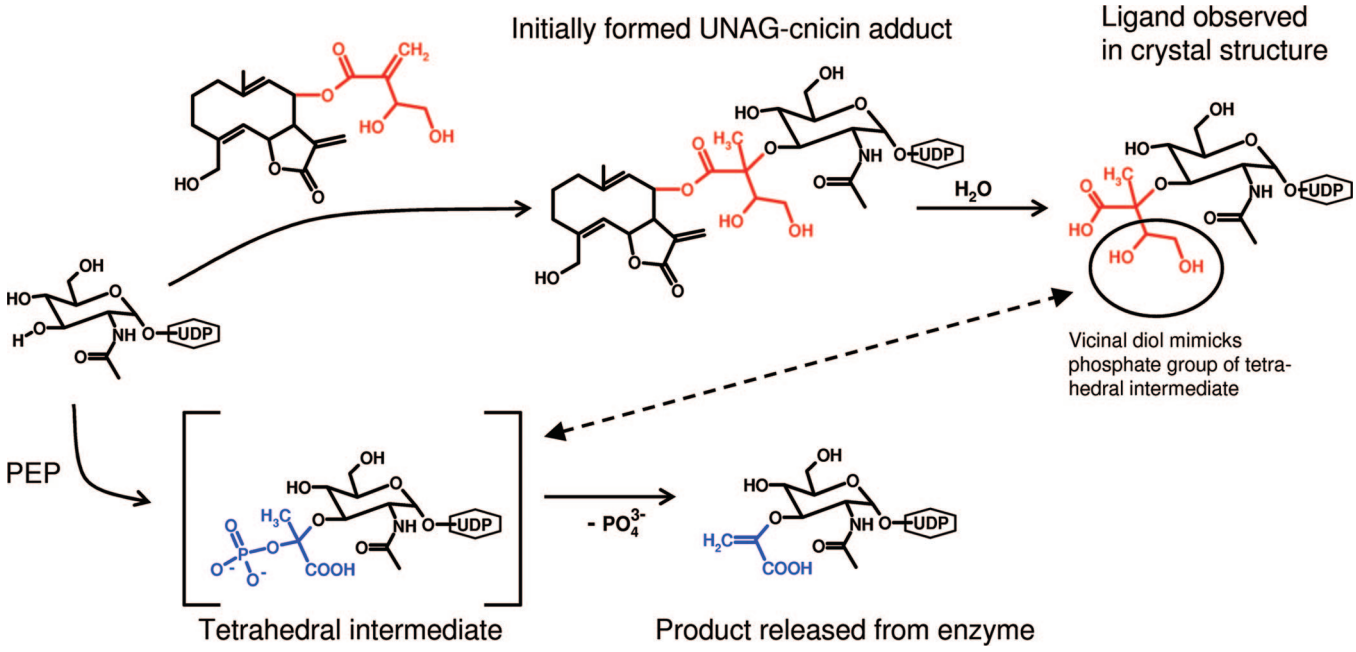

Figure 3. MurA-catalyzed reactions of UNAG with cnicin (top) and PEP (bottom). Note the structural similarity between the tetrahedral intermediate of the PEP reaction and the hydrolyzed UNAG-cnicin adduct observed in the crystal structure (dashed arrow).

of the native MurA reaction between PEP and UNAG. The best fit to the experimental electron density was obtained by the assumption of two alternative conformers of the cnicin side chain.

We assume that the following reactions have occurred (cf. Figure 3): (1) binding of UNAG and cnicin to MurA, partial closure of the active site; (2) MurA catalyzes the formation of the "initial" UNAG−cnicin adduct, which is a potent inhibitor of MurA; (3) upon storage of the MurA−UNAG−cnicin complex, the ester function hydrolyzes and the macrocyclic part of cnicin is eliminated. This is the status seen in the X-ray structure. Obviously, the removal of the cnicin macrocycle is necessary for the Cys115 loop to assume a conformation that is very similar to the MurA−fos structure, and the latter conformation is needed for crystal growth under these conditions. The considerable steric bulk of the cnicin macrocycle in the initial, modified UNAG molecule will hinder the complete closure of the Cys115 loop. Most probably, the slow growth of MurA−cni crystals represents the time necessary for the hydrolytic removal of the cnicin macrocycle.

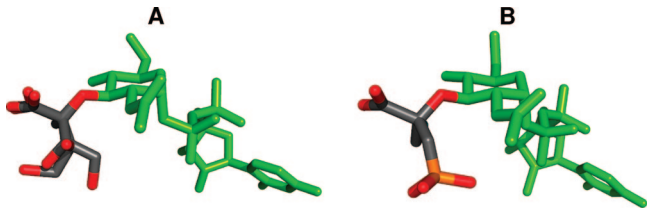

Figure 4. Comparison of the structures of (A) the UNAG−cnicin adduct and (B) the fluorinated tetrahedral intermediate of the native MurA reaction (PDB code 1A2N). The diol moiety of the UNAG−cnicin adduct imitates the phosphate unit of the tetrahedral intermediate.

Mechanistic Interpretation. The structure of the MurA−cni complex reveals that the enzyme MurA catalyzes the formation of its own, noncovalent inhibitor which resembles the tetrahedral intermediate (THI) of the "regular" physiological reaction. The formed UNAG−cnicin adduct exhibits evident similarity to the tetrahedral intermediate of the native MurA reaction: its vicinal diol function imitates the phosphate group of the THI (see Figure 4). Because of the absence of the leaving phosphate group, the UNAG−cnicin adduct may suspend the opening of the closed conformer of MurA essential for product release. This kind of mechanism-based inactivation of MurA may imply a long-lasting interaction of the UNAG−cnicin adduct with the polar and highly conserved amino acids Lys22, Arg120, and Arg331 in the active site of MurA (see Figure 5). The trapped UNAG−cnicin adduct in the active site explains the observed time-dependency of the MurA inhibition by cnicin.[1] However, the lifetime of the initially formed MurA−cni complex remains unclear, in particular because of the slow growth of the crystals and the hydrolytic removal of the macrocycle. Hence, the single turnover of the enzyme in the presence of cnicin and the arrest of the enzyme in the closed conformer is to be verified, e.g., with the pure synthesized UNAG−cnicin adduct.

Catalytic Mechanism of the 1,3-Addition. Various biological effects of sesquiterpene lactones are explained by their reactivity toward cysteine thiols via the Michael reaction (1,4-addition). From a chemical perspective, the formation of an ether linkage between the α-carbon of a Michael-reactive methylene function and the glucosamine alcohol function demonstrates an unusual and remarkable reaction. This is even more surprising in light of the fact that MurA contains an exposed cysteine residue (Cys115) within the active site, which we expected to be the nucleophilic target of cnicin and the other MurA inhibitors reported before in a 1,4-addition. 1,3-Additions (or anti-Michael) such as the one observed here are favored if electron-withdrawing groups like *nitro*-substituents are present at the *β*-carbon atom.[10,11] The most active compounds cnicin and cynaropicrin possess an alcoholic hydroxyl group close to the α,*β*-unsaturated carbonyl function. However, the electron-withdrawing potency of a hydroxyl group is undoubtedly not strong enough to reverse the polarity of the carbon−carbon double bond.

The active site of MurA involves numerous positively charged residues: four arginines (Arg91, Arg331, Arg120, and Arg371) and one lysine (Lys22). The pronounced ionic interactions with the positively charged guanidinium moieties of Arg331 and Arg120 that are positioned in vicinity to the *β*-carbon could play an important role for the addition of a nucleophile to the α-carbon. This unique environment may influence the electron density at the *β*-carbon, which could lead to a reversed regioselectivity of nucleophilic addition to the α,*β*-unsaturated carbonyl function. Electrostatic interactions in the active site of MurA are likely to be a major cause for the reverse addition (Figure 5). Furthermore, it can be hypothesized that the attacking nucleophile is the more reactive alcoholate of UNAG, which may have transferred a proton to Lys22, whose importance for the catalytic process has been described before.[12−15]

Cys115Asp Mutation. The *E. coli* Cys115 → Asp mutant MurA is not inhibited by cnicin,[1] either because it is unable to form the UNAG−cnicin adduct or the mutant enzyme is not sensitive to inhibition by the UNAG−cnicin adduct. Catalysis of the addition of the cnicin side chain may be favored in the presence of a cysteine, which can function as nucleophile and promote covalent catalysis. From this perspective, the formation of an initially covalent cnicin complex with Cys115 is possible

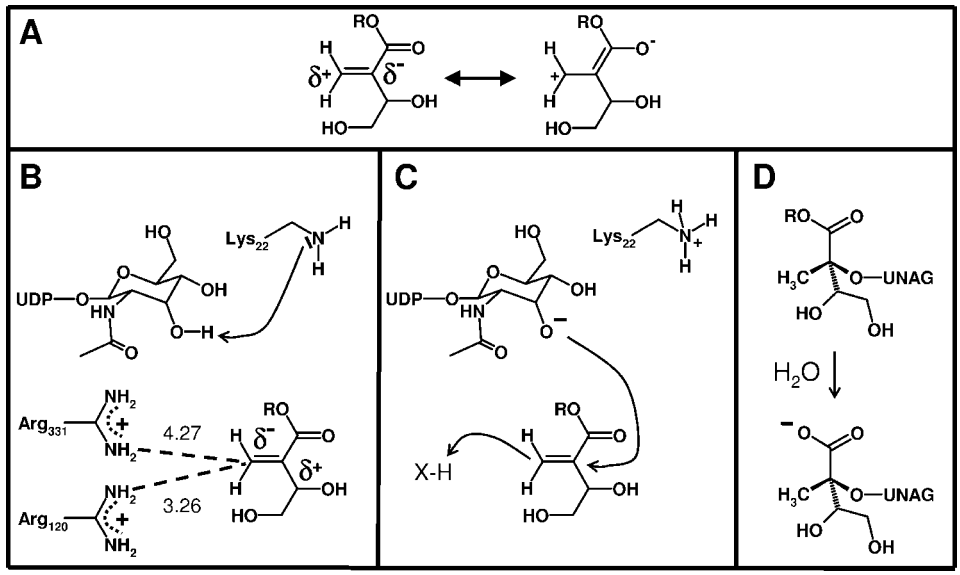

Figure 5. Proposed mechanism of the 1,3-addition. (A) "Ground state" of the cnicin side chain. *R* = macrocyclic lactone, cf. Figure 3. (B,C) The electrostatic effects of Arg120 and Arg331 may reduce the negative partial atomic charge at the α-carbon, thereby facilitating the addition of UNAG at this position. The attacking nucleophile could be the more reactive alcoholate of UNAG after proton transfer to Lys22. The proton (X-H) probably originates from a water molecule (WAT258 in the X-ray structure) that is positioned near the two arginines. The numbers are distances in Å as observed in the X-ray structure. (D) Hydrolytic removal of the macrocyclic part of cnicin within hours to days.

Table 1. Data Collection and Refinement Statistics

data collection and processing	
beamline	PX12 (DESY)
detector	MarCCD
wavelength (Å)	1.03317
temperature (K)	100
space group	P1
cell dimensions	
a (Å)	65.1
b (Å)	80.4
c (Å)	84.9
α (deg)	108.7
β (deg)	111.1
γ (deg)	101.2
maximum resolution (Å)	2.05
completeness (%)[a]	93.8 (93.2)
no. unique observations	84171
average redundancy[a]	1.9 (1.9)
$\langle I/\sigma(I)\rangle$[a]	7.5 (2.7)
R_{sym}(%)[a,b]	7.1 (29.5)
B-factor from Wilson plot (Å^2)	31.7

refinement statistics	
resolution range (Å)	19.14 − 2.05
$R_{working}$ (%)[a,c]	19.1 (23.9)
R_{free} (%)[a,d]	27.1 (31.8)
Ramachandran plot regions (%)[e] favored/allowed/generous	92.6/6.7/0.7 (Asi67ABCD, Ser349ABCD)
rmsd bond lengths (Å)	0.016
rmsd bond angle (deg)	1.71
mean *B*-factor (Å^2)[f]	
backbone (A/B/C/D)	9.5/9.5/9.4/9.1
side chain (A/B/C/D)	11.2/11.2 /11.2/10.6
cofactor (A/B/C/D)	2.7/2.5/2.7/4.3
water (932 molecules)	15.5

[a] Values in parentheses are for the high-resolution bin (2.1−2.05 Å). [b] $R_{sym} = 100 \times \sum_h\sum_i |I_i(h) - \langle I_i(h)\rangle| / \sum_h\sum_i I_i(h)$, where $I_i(h)$ is the *i*th measurement and $\langle I_i(h)\rangle$ is the mean of all measurements of $I(h)$ for Miller indices *h*. [c] $R = \sum(||F_{obs}| - k|F_{calc}||)/\sum|F_{obs}|$ where F_{obs} and F_{calc} are observed and calculated structure factor amplitudes, respectively. [d] R_{free} value is the *R* value obtained for a test set of reflections, consisting of a randomly selected 5% subset of the diffraction data (4202 reflections), not used during refinement.[26] [e] Calculated using the program PROCHECK.[27] The residues in the generously allowed regions are given in parentheses. Residue Asi67 is a L-iso-aspartate and Ser349 positioned in a tight *β*-turn. Both residues are well defined by electron density. [f] The four protein molecules of MurA within the asymmetric unit are labeled with chain IDs A, B, C, and D, respectively. The *B*-values were taken after TLS refinement.

in analogy to the covalent catalysis in the addition step of the natural ligand PEP.[16−18]

Conclusion

The X-ray structure and the underlying mechanism could represent a novel starting point for the development of antiinfectives targeting MurA. Relevant aspects for drug design are, in particular: (1) Cnicin and the other SLs are combinations of a reactive group (the unsaturated side chain) that is coupled to the macrocyclic lactone, which probably modulates pharmacokinetic aspects such as permeation into the bacterial cell. (2) Within the bacterial cytoplasm, the SLs are processed as false substrates by MurA and form adducts with UNAG. The end point of the reactions between SLs, UNAG, and MurA is observed in the crystal structure. (3) The structure of the UNAG−cnicin adduct is similar to the putative transition state of the natural MurA reaction. The replacement of the bridging oxygen of the tetrahedral intermediate by a carbon prevents the collapse of the transiently formed, unstable tetrahedral intermediate during the catalytic cycle.

The next steps will be to explore the antibiotic potential of preformed sugar and UNAG derivatives that resemble the transition state analogue observed here. Such compounds may have the additional potential to inhibit the Cys115Asp mutant of the enzyme. Furthermore, we will address the question whether the reactivity of the unsaturated ester side chain can be fine-tuned in such a way as to generate analogues with increased 1,3 reactivity and, hence, an even pronounced tendency to form the unusual product described here. Such compounds will, in addition, be less prone for (inactivating) 1,4 reactions, which inevitably occur in the medium outside the bacterial cell, than the SLs of natural origin.

Experimental Section

UNAG and PEP were purchased from Sigma-Aldrich (Deisenhofen, Germany). Cnicin was isolated from *Cnicus benedictus* L.[1] Recombinant *E. coli* MurA was produced in *E. coli* BL21(*λ*DE3) and purified by chromatographic techniques as reported.[1] MurA for the crystallization of the MurA−cni complex was obtained as described in ref 9. Crystals were obtained at 15 °C by vapor diffusion technique in 20 *μ*L sitting drops. One droplet consisted of 10 *μ*L of protein solution (10 mg/mL) containing 2.5 mM UNAG and 5 mM cnicin and 10 *μ*L of reservoir solution of 30% polyethylene glycol 4000, 100 mM Tris, pH 8.5. Crystals were grown over 1 mL of reservoir solution sealed with clear sealing tape. DTT was omitted during the incubation period of the inhibitor cnicin and the substrate UNAG before setting up the crystals.

X-ray Crystallography. Diffraction data of a flash-frozen crystal were recorded at 100 K using the EMBL beamline PX12 at the DORIS storage ring of DESY (Hamburg, Germany). Data were processed and scaled with XDS/XSCALE.[19,20] The MurA structure published by Skarzynski and co-workers (PDB code 1UAE) was used without water molecules as a starting model for molecular replacement using MOLREP[21] as implemented in the CCP4 program suite.[22] Refinement was performed using REFMAC5[23] in combination with TLS refinement[24] defining each of the four protein molecules of the asymmetric unit as one individual TLS group. Intermittent cycles of model building were done with the program COOT.[25] Evaluation of the progress of refinement was performed based on the R_{free} value,[26] electron density correlation, and quality of stereochemistry as analyzed by PROCHECK[27] (Table 1). The X-ray structure is available in the protein data bank PDB with entry-code 2Z2C. Representations of the protein structure were produced with PyMol.[28]

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