

The Active Principle of Garlic at Atomic Resolution*

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Despite the fact that many cultures around the world value and utilize garlic as a fundamental component of their cuisine as well as of their medicine cabinets, relatively little is known about the plant's protein configuration that is responsible for the specific properties of garlic. Here, we report the three-dimensional structure of the garlic enzyme alliinase at 1.5 Å resolution. Alliinase constitutes the major protein component in garlic bulbs, and it is able to cleave carbon-sulfur bonds. The active enzyme is a pyridoxal-5'-phosphate-dependent homodimeric glycoprotein and belongs to the class I family of pyridoxal-5'-phosphate-dependent enzymes. In addition, it contains a novel epidermal growth factor-like domain that makes it unique among all pyridoxal-5'-phosphate-dependent enzymes.

Garlic (*Allium sativum*) has been known and utilized as a spice and herbal remedy for more than 4000 years. Most likely originating from Central Asia, the plant made its way via the Old Sumerians, the Egyptian high cultures, the Greeks, and the Romans into modern European and American cuisine, pharmacy, and culture. Today, a myriad of pharmacological properties are attributed to garlic or its ingredients, ranging from blood lipid level and blood pressure lowering and inhibition of blood clotting to antiviral, antifungal, and antimicrobial activities and even cancerostatic effects (1–4). Although these pharmacological effects are little understood, it is clear that most of them rely on sulfur-containing garlic components. Many of the active compounds have been identified in garlic and other *Allium* species and found to belong to one of three groups: dithiines, allyl sulfides, and ajoenes (Fig. 1A). Their organic chemistry has been studied (2), and in some cases their therapeutic effects have been elucidated (4). All of these compounds originate from thiosulfonates such as alliin (Fig. 1B), which in turn are produced by the action of the enzyme alliinase (EC 4.4.1.4) on cysteine derivatives.

Garlic alliinase was first described in 1947 (5). It is a homodimeric enzyme of 2×448 amino acid residues and a total molecular weight of 103,000. The enzyme contains one pyridoxal-5'-phosphate (PLP)¹ cofactor per subunit and belongs to the

class I of PLP-dependent enzymes (6). With its C-S lyase activity, alliinase is able to cleave the C_β-S_γ bond of sulfoxide derivatives of cysteine to produce alliin (Fig. 1B). Because of the vacuolar location of the enzyme and the presence of its substrate in the cytosol, the alliinase/alliin system has been discussed as a primitive defense mechanism of the plant (7). Based on multiple sequence alignments of various alliinase sequences, the presence of a unique epidermal growth factor (EGF)-like domain was proposed (8). The protein also contains four putative N-glycosylation sites at Asn¹⁹, Asn¹⁴⁶, Asn¹⁹¹, and Asn³²⁸ (9). Recently, diffraction quality crystals of the natural form of the enzyme have been obtained (8, 10).

Here, we report the three-dimensional structure of garlic alliinase at 1.5 Å resolution. Alliinase is only the second garlic protein for which a structure determination has been carried out. We confirm the presence of the proposed EGF-like domain, rationalize the reaction mechanism, and provide some explanation for the substrate selectivity of alliinase.

EXPERIMENTAL PROCEDURES

Protein Production and Crystallization—Alliinase was purified from garlic bulbs as described previously (8). Well-diffracting crystals were grown from an ammonium sulfate solution in space group P2₁2₁2₁, with unit cell dimensions $a = 68.4$ Å, $b = 101.1$ Å, $c = 155.7$ Å, $\alpha = \beta = \gamma = 90^\circ$, one alliinase homodimer per asymmetric unit, and a solvent content of 50% (8). A gold derivative was prepared by co-crystallization of alliinase with 1.0 mM KAuCl₄.

Diffraction Data Collection, Structure Determination, and Refinement—The structure was determined using the single isomorphous replacement with anomalous scattering (SIRAS) method. The native data set was collected to a resolution of 1.53 Å at the EMBL beamline BW7B (DESY, Hamburg, Germany) and the gold derivative data set to a resolution of 2.1 Å at the x-ray diffraction beamline at the ELETTRA synchrotron (Trieste, Italy). Three gold sites were identified from an anomalous difference Patterson synthesis. Phases were calculated using the program MLPHARE (11) and improved by solvent flattening using the program DM (11). The figures-of-merit of the phases belonging to the correct hand were 0.33 and 0.73 before and after solvent flattening, respectively. The initial structure model was built using the ARP/wARP procedure (12), and structure refinement was carried out using the program REFMAC version 4 (11).

Structure Comparisons—Structural homology searches against other known structures were performed using the DALI server (13). The programs ALIGN (14) and LSQKAB (11, 15) were used to calculate the three-dimensional structural alignments and the root mean square differences between the aligned structures.

RESULTS AND DISCUSSION

The Overall Structure—The refined model of alliinase consists of residues 2–425 in subunit A, 2–427 in subunit B, 4 sugar chains, 830 water molecules, 2 chloride ions, and 11 sulfate ions as well as 2 partially occupied HEPES buffer molecules and 1 partially occupied aminoacrylate (AA) moiety. With R and R_{free} values of 19.3 and 22.1% (Table I) to a

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The atomic coordinates and structure factors (code no. 1LK9) have been deposited in the Protein Data Bank, Research Collaborator for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>).

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¹ The abbreviations used are: PLP, pyridoxal-5'-phosphate; aAT, ar-

omatic amino acid-aminotransferase; AA, aminoacrylate; ACC, 1-aminocyclopropane-1-carboxylate; EGF, epidermal growth factor.

resolution of 1.53 Å, it can be considered well refined. An example for the quality of the final electron density is shown in Fig. 2. The quaternary structure of alliinase is a dimer consist-

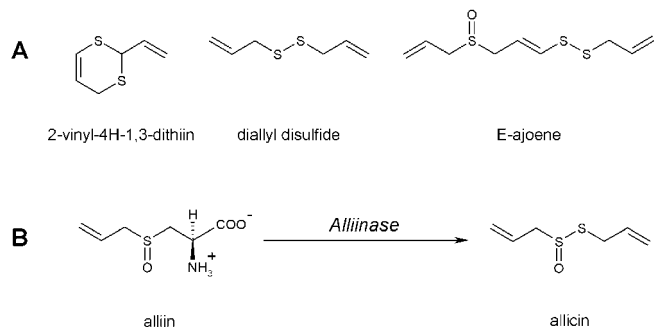


FIG. 1. *A*, therapeutically active sulfur compounds from garlic; a representative for each of the three substance classes (allyl sulfides, dithiines, and ajoenes) is shown. *B*, the enzymatic reaction catalyzed by alliinase.

ing of two identical subunits related by a rotation of 180° (Fig. 3) with a root mean square deviation between the two subunits of the homodimer of 0.36 Å based on the superposition of all 424 C α -pairs. Each alliinase monomer consists of three distinct domains, a unique N-terminal domain, the central PLP-binding domain, and the C-terminal domain. Comparisons of alliinase with 1-aminocyclopropane-1-carboxylate (ACC) synthase (16) as a representative for C-S lyases and aromatic amino acid-aminotransferase (aAT) (17) as a typical aminotransferase show that the overall folds of the central and the C-terminal domains of the three enzymes are similar (Fig. 4). The seven-stranded mixed β -sheet in the central domain and the three-stranded antiparallel β -sheet in the C-terminal domain flanked by three α -helices all on one side are present in all three cases. Three-dimensional alignments with the open (18) and the closed form of chicken mitochondrial aspartate aminotransferase (19) reveal that the conformation of alliinase is more akin to the closed than to the open form of aspartate aminotransferase.

The EGF-like Domain—A peculiar and distinguishing fea-

TABLE I
Data collection, phasing, and refinement statistics

	Native	KAuCl ₄ derivative
Data collection and processing statistics		
X-ray source	BW7B (DESY)	ELETTRA Trieste
Wavelength (Å)	0.842	0.855
Detector	MAR345	MARCCD
Resolution range (Å) ^a	20–1.53 (1.56–1.53)	33–2.10 (2.15–2.10)
Observed reflections	928,820	483,408
Unique reflections	160,747	64,801
Redundancy	5.8	7.5
Completeness (%) ^a	98.9 (99.6)	99.8 (99.5)
R_{merge} (%) ^{a,b}	5.7 (50.8)	8.9 (33.0)
R_{rim} (%) ^{a,c}	6.0 (59.3)	9.6 (35.4)
R_{pim} (%) ^{a,d}	2.2 (29.5)	3.5 (12.8)
R_{anom} (%) ^{a,e}		5.3 (13.8)
B-factor from Wilson plot (Å ²)	20.7	22.9
Phasing statistics		
Resolution range (Å)		20–2.10
$R_{\text{to-native}}$ (%)		31.5
No. of heavy atom sites		3
Phasing power (cen/ac) ^f		0.89/1.01
Figure of merit		0.334
R_{cullis} (%) (cen/ac)		83.0/86.0
Refinement statistics		
Resolution range (Å)	20–1.53	
Total no. of atoms	7,996	
No. of protein atoms	6,914	
R_{cryst} (%)	19.3	
R_{free} (%)	22.1	
Rmsd from ideality		
Bonds (Å)	0.011	
Angles (°)	2.2	
Ramachandran plot		
% in most favored regions	90.8	
% in allowed regions	8.9	
% in generously allowed regions	0.3	
% in disallowed regions	0.0	
Average B-factor (Å ²)		
Main chain	22.4	
Side chain	25.9	
PLP	22.6	
Amino acrylate	23.7	
Carbohydrates	44.9	
Chloride	18.9	
Sulfates	40.9	
Hepes	26.1	
Water molecules	34.9	

^a The numbers in parentheses refer to the outermost resolution shell.

^b $R_{\text{merge}} = 100 \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$.

^c $R_{\text{rim}} = 100 \sum_{hkl} (N/(N-1))^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$.

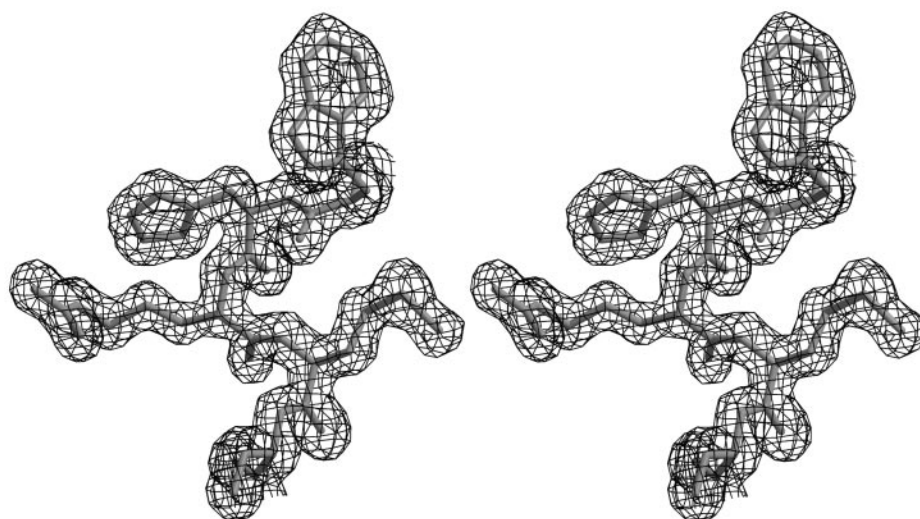
^d $R_{\text{pim}} = 100 \sum_{hkl} (1/(N-1))^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$.

^e $R_{\text{anom}} = 100 \sum_{hkl} |I(hkl) - I(-h-k-l)| / \sum_{hkl} \langle I(hkl) \rangle$.

^f Phasing power for centric (cen) and acentric (acen) reflections: $\langle F_H \rangle / \langle |F_{PH} - |F_P + F_H|| \rangle$.

^g $R_{\text{cullis}} = 100 \sum_{hkl} |F_{PH} - |F_P + F_H|| / \sum_{hkl} |F_{PH} - F_P|$.

FIG. 2. Stereo representation of the $(2F_o - F_c)$ -electron density of the segment Trp⁸⁷-Ser⁹¹ of the A-subunit of alliinase contoured at 1.2σ . The figure was prepared using the programs BOBSCRIPT (27) and RASTER3D (28).



A

B

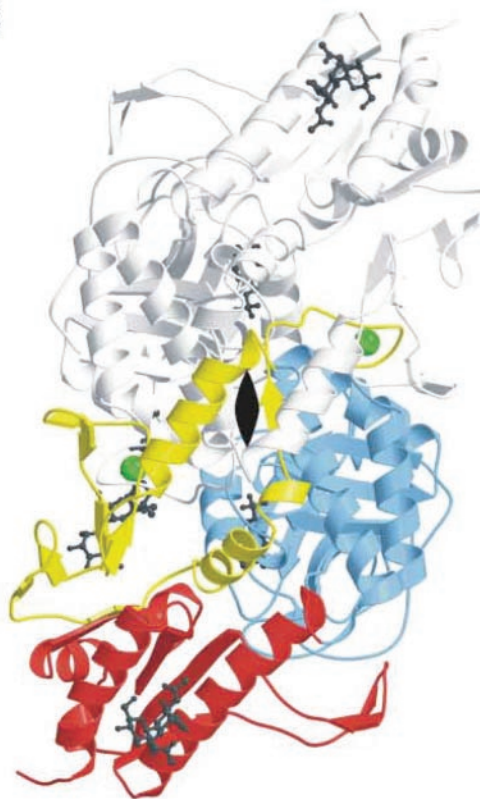
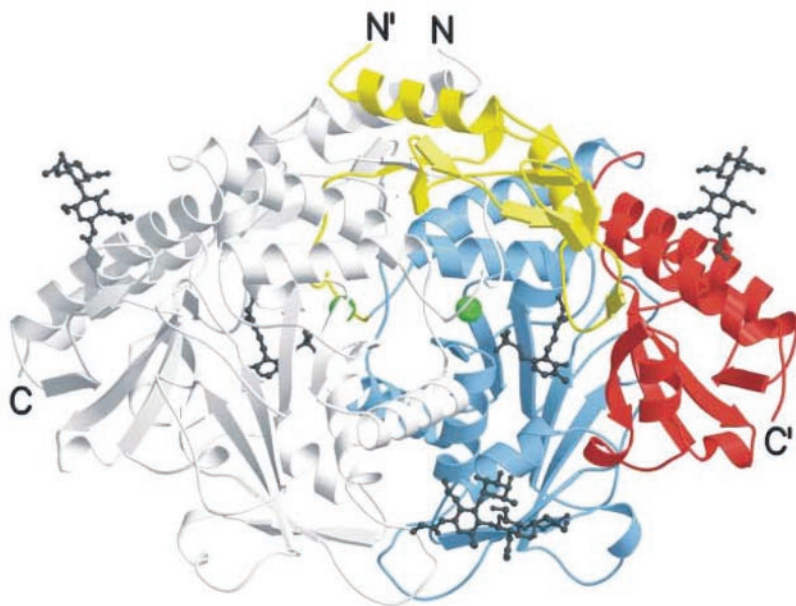


FIG. 3. The overall structure of the alliinase homodimer. One monomer is represented in color: N-terminal domain (residues 2–100) including the EGF-like domain, *yellow*; central domain (residues 101–310), *blue*; C-terminal domain (residues 311–427), *red*. The PLP cofactors and the sugar moieties are represented as *black ball-and-stick* models, the chloride ions as *green spheres*. The figure was prepared using the programs MOLSCRIPT (29) and RASTER3D (28). In (A), the 2-fold axis of the dimer is vertical in the paper plane, whereas in (B) the view is down the 2-fold axis. The typical S-shaped structure of the dimer is clearly discernible.

ture of the alliinase structure is the presence of an EGF-like domain in its N-terminal part (Fig. 5A). EGF-like domains are small disulfide-rich structures (20) that often constitute modules for binding to other proteins. Although they occur frequently in animal proteins, they are rather uncommon in plant proteins. When they do occur, they are usually found in the extracellular portion of membrane-bound or secreted proteins. Alliinase constitutes the first example of a catalytic domain fused to an EGF-like domain in a plant enzyme. Comprised of 47 amino acids, this domain ranges from Glu¹³ to Ala⁵⁹ and contains six cysteine residues arranged in a disulfide pattern

of the type (C1-C2, C3-C5, C4-C6). This pattern is different from the one (C1-C3, C2-C4, C5-C6) found in the canonical EGFs. The first and second disulfide bond of alliinase corresponds to the second and third of canonical EGFs (Fig. 5B). A search for structural homologues revealed that the closest known relative is the heparin-binding epidermal growth factor-like growth factor, also known as the diphtheria toxin receptor (21). A superposition of the two molecules is shown in Fig. 5B. The function of this EGF-like domain in alliinase is unclear. One may speculate that it is a binding site for other proteins or the docking site for a hypothetical alliinase receptor. An inter-

FIG. 4. **Comparison of garlic alliinase with other PLP-dependent enzymes.** Shown are garlic alliinase (left), ACC synthase from apple (PDB code 1B8G, Ref. 16) (center), and aAT from *Pyrococcus horikoshii* (PDB code 1DJU, Ref. 17) (right). A, topology diagrams: the spheres and triangles represent α -helices and β -strands, respectively. The small symbols represent α - or 3_{10} -helices containing four to seven residues and β -strands of three residues. B, MOLSCRIPT diagrams (29) of the monomers of the three enzymes in the same orientation. The overall root mean square deviations between alliinase and ACC synthase and aAT are 1.96 and 1.93 Å based on 300 and 285 aligned C $_{\alpha}$ pairs, respectively.

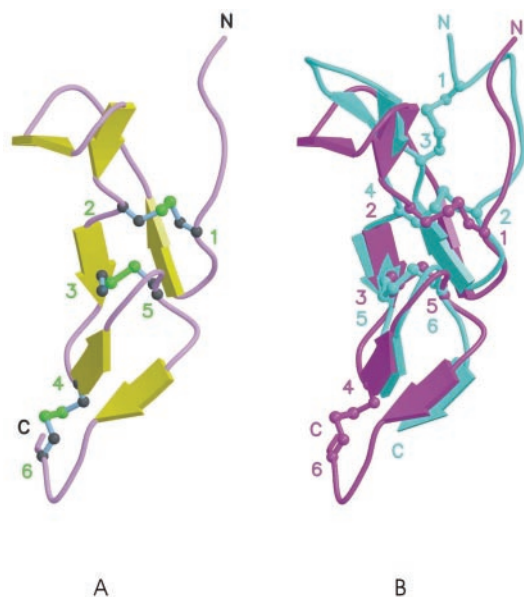
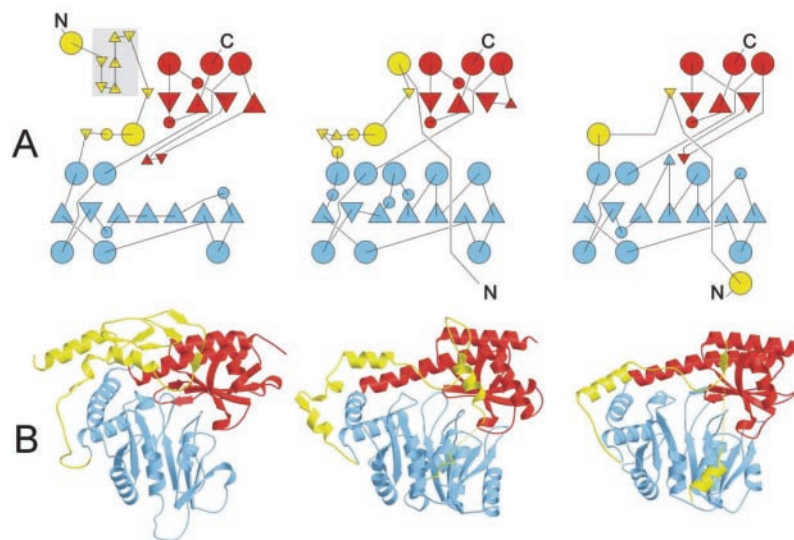


FIG. 5. **The EGF-like domain of alliinase from garlic.** A, MOLSCRIPT diagram (29) of the EGF-like domain of alliinase (amino acid residues 13–59). B, superposition of the alliinase EGF-like domain with the heparin-binding EGF-like growth factor (amino acid residues 107–147), which is also the receptor for diphtheria toxin (PDB entry 1XDT, Ref. 21). The overall root mean square deviation is 1.88 Å based on 37 aligned C $_{\alpha}$ pairs.

esting observation along this line is that garlic consumption leads to the appearance of anti-alliinase antibodies in human blood serum (22). This seems to imply that alliinase must be resorbed into the circulatory system with its three-dimensional structure still intact.

The Chloride Binding Loop—A strong peak in the anomalous difference electron density map revealed the presence of a chloride ion bound to the loop 92–100 in each of the subunits. The chloride ion is hydrogen-bonded to three main-chain amide NH groups (Phe⁹⁴, Ser⁹⁸, Phe¹⁰⁰) and one water molecule. It stabilizes the loop 92–100 (including a *cis*-peptide bond between Asn⁹⁵ and Pro⁹⁶) in a conformation that orients the aromatic amino acids Tyr⁹², Phe⁹³, and Phe¹⁰⁰ toward the active site of the neighboring subunit. These residues presumably bind the hydrophobic part of the substrate. The presence of chloride in this position provides a convincing explanation for the previously made observation that NaCl stabilizes the

enzymatic activity and enhances dimer stability (8, 23).

The Glycosylation Sites—Only two of the four predicted glycosylation sites per alliinase subunit (Asn¹⁴⁶ and Asn³²⁸) were observed to be utilized, although all four Asn residues are located at the surface of the molecule. The core sugar structure is Man(β 1–4)GlcNAc(β 1–4)[Fuc(α 1–3)]GlcNAc(β 1–N)Asn, which is quite typical for plant glycoproteins (Man: mannose; GlcNAc: N-acetylglucosamine; Fuc: fucose). Up to four sugar rings are visible in the electron density map at the four sites in the dimer. The sites at Asn¹⁴⁶ are located at the dimer interface, and the sugar chains bind to both subunits, thereby stabilizing the dimer. In contrast, the sites at Asn³²⁸ point into the solvent area and do not contact any protein atoms.

The Active Site (Cofactor Binding)—The active site of alliinase is located at the dimer interface and consists of residues from both subunits (Fig. 6). The PLP moiety is covalently bound to Lys²⁵¹ via a Schiff base, forming a so-called internal aldimine. The pyridine ring of the PLP is sandwiched between the aromatic ring of Tyr¹⁶⁵ and the isopropyl group of Val²²⁷, which forms a C–H $\cdots \pi$ hydrogen bond (24) to the PLP heterocycle (not shown in Fig. 6). Other PLP binding features include the hydrogen bonds from Asp²²⁵ to the pyridinium nitrogen and from Asn²⁰⁷ and Tyr²²⁸ to the phenolic oxygen atom of the PLP cofactor. The hydrogen bond formed by the amide side chain of Asn²⁰⁷ appears to be an especially interesting feature, because Asn²⁰⁷ belongs to the so-called “strained loop” (see below). The negative charge of the PLP-phosphate group is stabilized by the helical dipole of α -helix 132–143, by Arg²⁵⁹, and by four further hydrogen bonds involving the side chains of Thr¹³³, Thr²⁴⁸, Ser²⁵⁰, and Tyr⁹² of the neighboring subunit, a structural motif which has been termed the phosphate-binding cup (25). Taken together, all parts of the PLP molecule are held very tightly by the enzyme, reflecting the need to not lose the cofactor during the reaction cycle when the covalent bond between the enzyme and the cofactor is broken.

The Active Site (Substrate Binding)—Additional electron density in the active site of alliinase suggested the presence of other chemical entities (Fig. 7). The electron density was interpreted as HEPES molecules from the crystallization medium binding to both active sites of the dimer, albeit at partial occupancy. In addition, in one of the active sites an AA moiety was found to be covalently linked to the PLP cofactor but also at partial occupancy only. The AA most likely originated from the inhibitor *S*-ethyl-L-cysteine (SEC), which was present during the crystallization of alliinase (8). Apparently, SEC is

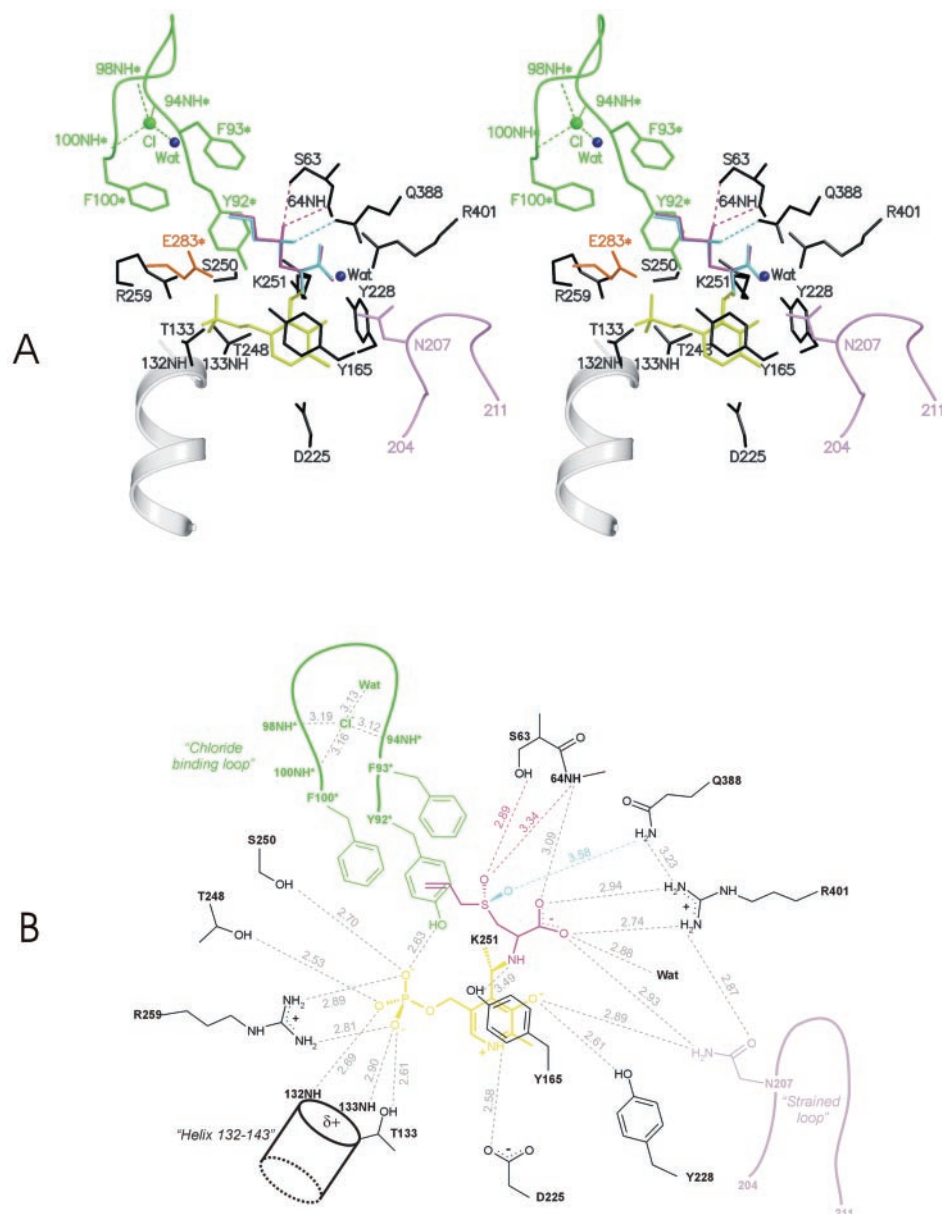


FIG. 6. **The active site of alliinase from garlic.** A, stereo diagram of all residues in hydrogen bonding distance to either the cofactor PLP (yellow) or the modeled substrate alliin. B, schematic diagram of the active site architecture. Both alliin configurations are shown: (+)-alliin, (magenta) and (–)-alliin, (cyan). Hydrogen bonding distances are given in Å.

not an inhibitor but a poor substrate. The current electron density is consistent with the presence of a 50:50 mixture of internal aldimine (Lys²⁵¹-PLP with HEPES present) and external aldimine (Lys²⁵¹ plus PLP-AA), although the formation of a geminal diamine (Lys²⁵¹-PLP-AA) cannot be ruled out. In any case, the observed architecture enabled us to model the real substrate alliin into the active site (Fig. 6). The carboxylate group (bound tightly by the guanidinium group of Arg⁴⁰¹), the C α , and the nitrogen atom of the AA moiety were assumed to occur in the identical positions in the substrate. The sulfoxide and the allyl group of alliin were then oriented so that the sulfoxide oxygen was close to a hydrogen bond donor. The only conformation without steric clashes turned out to be the one in which hydrogen bonds between the sulfoxide oxygen of a (+)-alliin and Ser⁶³-OH and the backbone amide NH of Gly⁶⁴ are formed (Fig. 6B). The allyl group would then be in close contact to the side chain of Tyr⁹² of the neighboring subunit. For (–)-alliin in the same conformation, the hydrogen bond to Ser⁶³-OH is not possible, but instead a weak hydrogen bond to the amide side chain of Gln³⁸⁸ could be formed (Fig. 6B). In these alliin conformations, the sulfoxide oxygens assume approximately

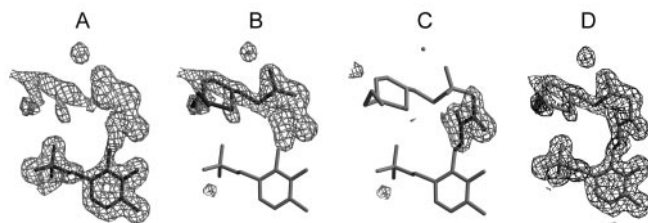


FIG. 7. **Interpretation of the electron density in the active site of subunit B of the alliinase dimer.** A–C, omit difference electron density contoured at a level of 3.0 σ superimposed on the successive interpretation of the density. A, PLP, HEPES/water, and AA were omitted for phase calculation. This electron density can be partly explained by the atoms of the PLP cofactor as indicated. B, HEPES/water and AA were omitted for phase calculation. The remaining density can be explained by the presence of a partially occupied HEPES molecule. Note that a water molecule binds tightly to two oxygen atoms of the sulfonyl group of HEPES. C, only AA was omitted for phase calculation. The remaining difference density can be explained by the presence of an AA moiety bound to the PLP ring. D, (2F_o – F_c) – electron density contoured at 1.0 σ . The figure was prepared using the programs BOBSCRIPT (27) and RASTER3D (28).

the same positions as the sulfone oxygen atoms in the bound HEPPES molecules, which lends further support to our model. In addition, the sulfoxide oxygens of alliin are relatively far from the carboxylate oxygens of Glu²⁸³ of the neighboring subunit (5.8 and 6.0 Å for (+)-alliin and 5.6 and 6.3 Å for (−)-alliin), thus minimizing electrostatic repulsion. These pieces of evidence, taken together, provide a possible explanation for the selectivity of alliinase toward (+)-alliin.

The Strained Loop Thr²⁰³ to Glu²¹¹—In both subunits of the dimer, a highly strained loop was observed to be involved in the binding of both the PLP cofactor and the substrate. This loop occurs between β -strands 3 and 4 of the central seven-stranded β -sheet in the PLP-binding domain and is located at the interface of the central PLP-binding and the C-terminal domains. It contains the two *cis*-peptide bonds, Ser²⁰⁴-Pro²⁰⁵ and Asn²⁰⁷-Pro²⁰⁸. With ω -angles of -4° and $+18^\circ$ in subunit A and -10° and $+25^\circ$ in subunit B, both these peptide bond conformations deviate significantly from planarity. In addition, the *trans*-peptide bonds Asn²⁰⁶-Asn²⁰⁷ ($\omega = 170^\circ$ and 168°) and Pro²⁰⁸-Glu²⁰⁹ ($\omega = -168^\circ$ and -164°) are also significantly non-planar, and Pro²⁰⁸ makes a van der Waals contact to Pro¹⁶³ of the neighboring loop, which is preceded by another peptide bond in *cis*-conformation. Because the side chain of Asn²⁰⁷ forms a hydrogen bond to the PLP cofactor (see above) and presumably to the substrate as well (Fig. 6), it may be that this strained conformation has some influence on the conformational change that is expected to happen during the reaction cycle. This change presumably involves a reorientation of the C-terminal domain relative to the central PLP-binding domain. Because of its location at the interface of the two domains, the strained loop is a prime candidate for triggering such a reorientation. In a mutational study in aspartate aminotransferase from *Escherichia coli* it could be shown that the *cis*-conformation corresponding to the Asn²⁰⁷-Pro²⁰⁸ peptide bond is retained when the Pro is replaced by an Ala, whereas the *cis*-conformation corresponding to the Ala¹⁶²-Pro¹⁶³ peptide bond is not (26). Furthermore, based on the reducibility of the PLP-Lys aldimine it was indicated that the mutation of Pro¹⁹⁵ (which corresponds to Pro²⁰⁸ in alliinase) to Ala may affect the open/closed equilibrium of the enzyme (26). This is in full accord with our hypothesis.

In conclusion, the elucidated structure of alliinase opens the door to a more rational understanding of garlic sulfur chemis-

try, and it may even provide a first step toward the elucidation of the reaction mechanism and the design of altered selectivities and therefore altered therapeutic features of garlic and other *Allium* plants.

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