Thiol-disulfide organization in alliin lyase (alliinase) from garlic (*Allium sativum*)

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Received 18 June 2008; Revised 16 September 2008; Accepted 7 October 2008

DOI: 10.1002/pro.10

Published online 2 December 2008 proteinscience.org

Abstract: Alliinase, an enzyme found in garlic, catalyzes the synthesis of the well-known chemically and therapeutically active compound allicin (diallyl thiosulfinate). The enzyme is a homodimeric glycoprotein that belongs to the fold-type I family of pyridoxal-5'-phosphate-dependent enzymes. There are 10 cysteine residues per alliinase monomer, eight of which form four disulfide bridges and two are free thiols. Cys368 and Cys376 form a S—S bridge located near the C-terminal and plays an important role in maintaining both the rigidity of the catalytic domain and the substrate-cofactor relative orientation. We demonstrated here that the chemical modification of alliinase with the colored —SH reagent *N*-(4-dimethylamino-3,5-dinitrophenyl) maleimide yielded chromophore-bearing peptides and showed that the Cys220 and Cys350 thiol groups are accessible in solution. Moreover, electron paramagnetic resonance kinetic measurements using disulfide containing a stable nitroxyl biradical showed that the accessibilities of the two —SH groups in Cys220 and Cys350 differ. Neither enzyme activity nor protein structure (measured by circular dichroism) were affected by the chemical modification of the free thiols, indicating that alliinase activity does not require free —SH groups. This allowed the oriented conjugation of alliinase, via the —SH groups, with low- or high-molecular-weight molecules as we showed here. Modification of the alliinase thiols with biotin and their subsequent binding to immobilized streptavidin enabled the efficient enzymatic production of allicin.

Keywords: alliinase; EPR; chemical modification; free thiols

Additional Supporting Information may be found in the online version of this article.

Abbreviations: b-S-alliinase, biotinyl-S-alliinase; CD, circular dichroism; DDPM, *N*-(4-dimethylamino-3,5-dinitrophenyl) maleimide; DTNB (Ellman's reagent), 5,5'-dithio-bis-(2-nitrobenzoic acid); DTP, 4,4'-dithiodipyridine; DTT, dithiothreitol; EGF, epidermal growth factor; EPR, electron paramagnetic resonance; NTB, nitrothiobenzoate; PBS, phosphate-buffered saline; PLP, pyridoxal-5'-phosphate; PLP-AA, PLP-amino acrylate; TEMPO, 2,2,6,6-tetramethyl-1-piperidinyloxy.

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Introduction

Alliinase (Cys sulfoxide lyase, alliin lyase, C-S lyase; EC 4.4.1.4) from garlic (*Allium sativum*) is an enzyme that uses pyridoxal-5'-phosphate (PLP) as a cofactor to catalyze the conversion of a nonprotein amino acid alliin (+) *S*-allylcysteine sulfoxide) to allicin (diallyl thiosulfinate), pyruvate, and ammonia, as shown in the following scheme:

Cloning and sequencing of alliinase shows that the enzyme subunit has a molecular mass of 51,500 and

consists of 448 amino-acid residues.^{1,2} Alliinase has been crystallized and its three-dimensional structure solved.^{3–6} The enzyme is a homodimeric glycoprotein belonging to the fold-type I family of PLP-dependent enzymes. Allicin, a product of the enzymatic reaction of alliinase with alliin, is the well-characterized, biologically active compound of garlic. It is responsible for the pungent odor and for a variety of biological effects attributed to garlic preparations, including antimicrobial, anticancer, antiatherogenic, and other activities.^{7–9} Most of the effects of allicin, a small, highly reactive, hydrophobic molecule, can be attributed to its ability to modify free —SH groups. Through its activated disulfide bond, —S(O)—S—, it reacts with various thiol-containing molecules, such as —SH groups of proteins^{10–15} or of low-molecular-weight compounds,¹⁶ thereby forming their *S*-allylmercaptoderivatives. Under natural conditions in garlic plant cells, the enzyme alliinase resides in microcompartments separated by thin membranes, and is thus physically kept apart from its substrate alliin. Crushing or injuring of a garlic bulb breaks down the compartmentalization and brings the enzyme and its substrate into contact, leading to allicin production (see scheme mentioned earlier). It is widely believed that this reaction contributes to the chemical defense of the plant. A number of studies have conclusively determined that direct contact of alliinase with its product allicin does not inactivate the enzyme.^{2,17–19} This could be interpreted to mean that alliinase does not contain free —SH groups, or alternatively, that modification of these groups is not important for its enzymatic activity.

Catalytic activity and stability of proteins that belong to the family of PLP enzymes frequently depend on their —SH groups of cysteine residues. For example, modification or substitution of the —SH groups of Cys390 in cytosolic aspartate aminotransferase from pig heart, or of Cys42 (situated near the active-site lysine) of *O*-acetylserine sulfhydrylase, dramatically changes the catalytic activity and efficiency of those enzymes.^{20,21} The —SH group of Cys-111 in Dopa decarboxylase is also located near the PLP-binding site and even though not essential for its catalytic activity plays a structural role in binding of PLP. This suggests that the cysteine residue is required for maintenance of the proper active-site conformation.²² In glutamate decarboxylase from *Escherichia coli*, concomitant with reactivation and reconstitution of the hexameric form is the burying of exposed cysteine residues.²³ A distinctive property of the branched chain aminotransferase is sensitivity to reducing agents. The enzyme contains a CXXC motif and belongs to the group of enzymes that can be regulated by their redox status.²⁴

Here we describe the role and arrangement of thiol-disulfides in alliinase. Previous amino-acid sequence analysis revealed 10 cysteine residues per alliinase subunit,² and X-ray crystal structure showed that six of these cysteine residues form three adjacent

disulfide bridges at the N-terminal part of the alliinase subunit.^{4,6} In the present study, we examined the configuration and reactivity of the remaining four cysteine residues and demonstrated that two free thiols are located far from the active site of the enzyme, and their modification does not affect the enzyme's structure and activity. Cys368 and Cys376 form a S—S bridge located close to C-terminus and has a role in maintaining both the rigidity of the catalytic domain and the substrate-cofactor relative orientation.

Results

Titration and identification of free thiols in alliinase

Incubation of native alliinase either with 4,4'-dithiodipyridine (DTP) or with 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) in the presence of 6*M* guanidine-HCl provided evidence for the existence of free cysteine residues in the alliinase molecule. Calculation of the ratio OD₃₂₄/alliinase for DTP and OD₄₁₂/denatured alliinase for DTNB yielded, respectively, 1.5 and 1.6 free thiol groups per the enzyme subunit. Preincubation with DTNB or with DTP did not result in any decrease of the initial alliinase activity. To identify the free cysteine residues, alliinase was modified by treatment with *N*-(4-dimethylamino-3,5-dinitrophenyl) maleimide (DDPM) and digested with trypsin, chymotrypsin, or pepsin as described in Materials and Methods. Peptides in digests containing the nitrophenyl chromophore were separated and detected on a 360-nm absorbance profile using reversed-phase HPLC. By analyzing the trypsin and chymotrypsin digests, we were able to identify a single (but different) Cys-containing peptide in each case. In the case of trypsin, it was a peptide containing a sequence HAVIKGX, where X corresponds to Cys220, and in the case of chymotrypsin the peptide contained the sequence XNYF, where X corresponds to Cys350. Treatment with pepsin made it possible to identify both of these free cysteine residues simultaneously in one digest. (see Fig. 1). These experimental findings (predating the alliinase structure determination) provided direct confirmation that the two free thiols contained in the alliinase molecule come from Cys220 and Cys350.

Electron paramagnetic resonance study of the alliinase —SH groups

Using electron paramagnetic resonance (EPR) spectroscopy, we examined the availability of the free —SH groups of alliinase for chemical modification with the disulfide containing the biradical •RS—SR•.^{25,26} The rate of the thiol-disulfide exchange reaction was monitored by EPR assay of the monoradical •R—SH released in this reaction, according to Eq. (1).

Figure 2 shows the increase in peak intensity of the EPR signal for the reaction between the biradical and

Figure 1. Reversed-phase HPLC of a pepsin digest of garlic alliinase. Alliinase was modified with DDPM, then denatured, carboxymethylated, and digested as described in Methods. The digest was placed in a Vydac Protein & Peptide C-18 column (4.6 mm $\times$ 250 mm), equilibrated with 0.1% trifluoroacetic acid, and eluted on a linear gradient of 0–80% acetonitrile. Elution was monitored by absorbances at 220, 280, and 360 nm. At least five peptides were detected on the 360-nm absorbance profile). Peaks 1–4 contain different peptides contained Cys220. As an example, the sequence of peak 3 was LRHAVIKGXKSI, where X was matched to the localization of Cys220, and the sequence of peak 5 was YXNYF, where X was matched to the localization of Cys350.

the native alliinase. These data demonstrated that the kinetics of modification occur at two different rates. Pretreatment of alliinase with *p*-chloro mercury benzo-

Figure 2. Kinetics of the nitroxyl biradical modification of alliinase. Peak intensity of the EPR spectrum of the monoradical component that was released into solution [as a result of the thiol–disulfide exchange between alliinase and the biradical; see Eq. (1)] was plotted as a function of time. The enzyme preparation (4 μ M) was added to a solution of the biradical (20 μ M) in PBS (pH 7.4).

Figure 3. EPR spectrum of the alliinase-biradical conjugate. The conjugate (12 mM, prepared as described in Methods) was measured in a PBS/glycerol (50/50) mixture at 120 K. EPR conditions: microwave power, 10 mW; modulation amplitude, 1.25 G; gain, 2×10^4 .

ate dramatically inhibited the modification kinetics (data not shown). Figure 3 shows the EPR spectrum of alliinase modified by the biradical (after 5 h of incubation followed by removal of the excess reagent by gel filtration) at 120 K. This spectrum is typical of a nitroxyl stable radical in a frozen solution. The degree of modification obtained by double integration of the EPR spectrum was 1.61 ± 0.15 per subunit of alliinase. To estimate the distance between two labeled cysteine residues, we used the empirical parameter d_1/d (see Fig. 3), which characterizes the dipole–dipole interaction between unpaired electrons of two nitroxyl groups, as proposed by Kokorin *et al.*²⁷ and commonly used to estimate the distance between two radicals covalently bound to proteins.^{28,29} In the absence of dipole–dipole interactions between the radicals, a value $d_1/d < 0.4$ is expected.^{27–29} The value obtained for d_1/d obtained in our experiment was 0.38, and the distance between the labeled cysteines Cys220 and Cys350 was estimated to be larger than 22 Å.

CD analysis of the modified alliinase

To determine whether the –SH modification of alliinase affects its folded structure in solution, we used CD spectroscopy. The CD spectra in the far UV (see Fig. 4) clearly show that the secondary structure is largely retained after modification of –SH groups by DTP and is almost identical to the CD spectra of native alliinase, indicating that the structure of the modified protein remains essentially unchanged.

Structural analysis of the cysteine network arrangement

Alliinase from garlic is a PLP-dependent fold-type 1 enzyme of the aminotransferase family.^{4,6} The enzyme's subunit consists of two major domains (see Fig. 5): the smaller, substrate-binding domain (domain

Figure 4. CD spectra in the far and near UV of native and DTP-modified alliinase. The solid line denotes native alliinase; the dashed line denotes the modified enzyme. Modification of alliinase by DTP was performed by titration with a 20-fold excess of the reagent over the protein concentration for 3 h at room temperature. Excess of DTP was then removed by gel filtration on a Sephadex G-25 column. Protein concentrations were 5 and 15 μ M in far and near UV, respectively.

1), and the larger central PLP-binding domain (domain 2). Previously, sequence analysis revealed the presence of 10 cysteine residues per subunit,² and the X-ray crystal structure elucidated the three-dimensional arrangement of all cysteine residues.^{4,6} Six cysteine residues were seen to be concentrated in the N-terminal part of alliinase domain 1. They form three disulfide bridges Cys20–Cys39, Cys41–Cys50, and Cys44–Cys57. Of the four remaining cysteine residues Cys368 and Cys376 form a disulfide bridge and Cys220 and Cys350 are free thiols. The Cys368–Cys376 disulfide bridge is located in the distal part of domain 1, close to the C terminus tightly locking the 368–376 Ω loop between two subdomains and is linked, through a network of interactions, to the PLP cofactor (see Fig. 6). Near the S–S bond between Cys368 and Cys376, Trp365 forms a π – π interacting aromatic pair with Tyr399. This π – π stacking, together with the disulfide bond, stabilizes the β -strand, thereby fixing the orientation of the nearby residue Arg401, found to be essential in its interactions via salt bridges to the PLP-amino acrylate (PLP-AA). Arg401 also interacts with Tyr363 through anion– π interaction of guanidine with the aromatic ring, and makes a hydrogen bond with Tyr228 (see Fig. 6). Arg401 makes a salt bridge and Tyr228 a hydrogen bond with a PLP-AA intermediate product⁶ (see Fig. 6).

It is notable that, in addition to the Cys368 and Cys376, the residues that participate in the discussed interactions, Tyr399, Trp365, Tyr363, Tyr228, and Arg401, are also highly conserved amongst alliinases (see Fig. 7). These interactions secure the relative orientations of PLP and carboxyl of a substrate, providing the correct positioning of a substrate and apparently playing a role in stabilizing the enzyme–substrate interaction.

As shown earlier, the enzyme subunit also contains two free thiols: Cys220 in the PLP-binding domain 2 and Cys350 in the C-terminal part of domain 1 (C7 and C8, respectively) (Figs. 5 and 8) located relatively far from the active site and from the substrate-binding area. As shown in Figure 8(A), Cys220 is located on the surface of the alliinase molecule, while Cys350 is in a more buried location but is still water-accessible. The free thiol groups of Cys220 and

Figure 5. Distribution of cysteines in a monomer of alliinase from garlic (*Allium sativum*). (A) The N- and C-termini of the alliinase monomer are indicated. The N-terminal and C-terminal parts of the small domain are colored in red, and yellow, respectively, and the catalytic domain is in blue. [O] PLP-AA are represented by red spheres. Cysteines are depicted as green spheres and indicated by one number for free cysteines and two numbers for bridged systems. (B) Dimeric structure of alliinase is depicted in the illustration. Yellow and magenta indicate monomers A and B, respectively. PLP groups are shown as red spheres. The dimer is rotated 180° around a vertical axis with respect to A. Free cysteines are shown as green spheres. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 6. S—S bridge formed by Cys368 and Cys376 in the C-terminal part of alliinase locks tightly 368–376 Ω loop. Series of interactions are formed between the Ω loop along two short antiparallel β -strands with PLP on the opposite flank. Interacting residues are Tyr399, Trp365, Tyr363, Arg401. Tyr363 interacts with Arg401 through anion- π interaction of guanidine with the aromatic ring and forms a hydrogen bond with Tyr228. Arg401 forms a salt bridge and Tyr228 forms a hydrogen bond with PLP-AA, respectively. Relevant distances are shown. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Cys350 have different relative orientations with respect to each other [Fig. 8(B)], which might affect their chemical modification rates. Distances between all the cysteines involved in disulfide bonds and the free thiols in the alliinase dimer range between 15 and 68 Å, and do not allow rearrangement of disulfide bonds in the native state.

Preparation of *b*-S-alliinase through —SH groups and its interaction with immobilized streptavidin

The results described earlier confirmed the presence of two free —SH groups in the alliinase subunit and four —SH groups in the dimeric holoenzyme molecule, which do not influence the enzyme's activity or structure. This finding allowed us to prepare alliinase con-

jugates via the —SH groups for further possible application of this enzyme. We prepared the alliinase biotinylated via its —SH groups with biotinyl-maleimide. Biotin residues were successfully introduced into alliinase, as shown by the modified protein ability to bind to immobilized streptavidin (see Fig. 9). The enzymatic production of allicin by the immobilized streptavidin-*b*-S-alliinase complex was very similar to that of the unmodified enzyme (see Fig. 9).

Discussion

The primary aim of this study was to investigate the accessibility and the reactivity of the free thiols in solution for the ultimate purpose of preparing a complex of biotin-modified alliinase and streptavidin that leaves unaltered alliinase activity for potential therapeutic applications. Despite the fact that location and organization of the cysteine residues in the garlic alliinase molecule were unambiguously resolved in the crystalline state, their structural significance was not examined in the previously published structural accounts^{4,6} nor were they analyzed with the respect to the solution behavior. The six cysteines located in the N-terminal part of domain 1 form three S—S bridges (Cys20—Cys39, Cys41—Cys50, and Cys44—Cys57) and adopt an EGF-like structure that most closely resembles diphtheria toxin receptor.⁴ We found that cysteine residues were distributed over both domains of the alliinase molecule. In the large PLP-binding domain 2, one cysteine residue (Cys220) was found to exist as a free thiol. Another free thiol (Cys350) is located at the C-terminal part of the enzyme's domain 1. Modification of cysteine residues by DDPM allowed us to obtain chromophore-bearing peptides and identify free alliinase thiols corresponding to Cys220 and Cys350. EPR kinetic measurements revealed that accessibilities of the two —SH groups differ and inspection of alliinase structure (4, 6) provides a rationale. The kinetics of chemical modification of alliinase via a thiol—disulfide exchange reaction with DTP and nitroxyl biradical indicate that both of the free —SH groups of the alliinase subunit can be modified without altering the enzymatic activity. Free —SH groups are known to play important structural and catalytic roles in a variety of enzymes. As an example, chemical modification of a nonconserved free Cys231 in acetylcholinesterase from *Torpedo californica* by the biradical $\cdot\text{RS—SR}\cdot$ or DTP transforms an entire molecule into the partially unfolded (molten globule) inactive state. In such cases, where the chemical modification induced unfolding of the protein, there was a concomitant complete disappearance of ellipticity in the near-UV of the CD spectrum ($\lambda_{\text{min}} = 280 \text{ nm}$) and protein inactivation.³⁰ In the case of alliinase, chemical modification of both free cysteine residues was found to leave both the secondary and the tertiary structure of the enzyme unchanged (see Fig. 4). This might be attributable to the marked thermodynamic and structural stability of

A	217.....227.....237.....247.....
Garlic	IKGCKSIYDM VYYWPHYTPI KYKADEDILL FTMSKFTGHS
Chinese onion	IKGCKSIYDM VYYWPHYTPI KYKADEDILL FTMSKFTGHS
Chives	IKGCKSIYDM VYYWPHYTPI KYKADEDIML FTMSKYTGHS
Shallots	IKGCKSIYDM VYYWPHYTPI KYKADEDIML FTMSKYTGHS
Bunching onion	IKGCKSIYDM VYYWPHYTPI KYKADEDIML FTMSKYTGHS
Onion	IKGCKSIYDM VYYWPHYTPI KYKADEDIML FTMSKYTGHS
Onion roots	IPGSLAVYDH ATYWPHYAPI KYASDEDIML FALSKYTGHS
Garlic roots	INGSLAIHDH VYYWPHYTPI AQPADEDIML FSMSKFTGHS
Chinese chives	IVGSTAIYDM VYYWPHFTPI TYKADEDIML FTMSKYTGHS
Giant onion	INGSKTIYDM VYYWPHYTPI KYKPEDIML YTMSKYTGHS

B	346.....356.....366.....376.....386.....396.....
Garlic	QSEYCNFYRR MRPPSPSYAW VNCEWEEDKD CYQTFQNGRI NTQSGVGFEA SSRYVRLSLI
Chinese onion	QSEYCNFYRR MRPPSPSYAW VKCEWEEDKD CYQTFQNGRI NTQSGVGFEA DSSYVRLSLI
Chives	QSEYCNFYRR MRPPSPSYTW VKCEWEEDKD CYQTFQNGRI NTQSGVGFEA GSRYVRLSLI
Shallots	QSEYCNFYRR MRPPSPSYAW VKCEWEEDKD CYQTFQNGRI NTQSGEGFEA GSRYVRLSLI
Bunching onion	QSEYCNFYRR MRPPSPSYAW VKCEWEEDKD CYQTFQNGRI NTQNGEGFEA GSRYVRLSLI
Onion	QSEYCNFYRR MRPPSPSYAW VKCEWEEDKD CYQTFQNGRI NTQNGEGFEA GSRYVRLSLI
Onion roots	-AEYCNFYQR IRNPSPTYGW LKCEWEEDTD CAAVLSNGKI LTQSGVLFEA SSRYARLSII
Garlic roots	-PQYCNFYFK VRDPSPSEGW LKCEWEQDTD CEALLRSAQI KTQSGVLFEA DSRVTRLSLI
Chinese chives	QSEHCNMYRM KRPPSPSYAW VRCNWPGEEN CSEVFKEGGI ITQDGPRFEA GSQYVRLSLI
Giant onion	QSEYCNFYFK MRPPSPSYAW VKCEWAEDKD CYQVFSQSGRI NTQGGAGFEA DSRVRLSLI

Figure 7. Alignment of segments of amino-acid sequences of alliinase from various sources. **(A)** The region from residue 217–257 in the garlic alliinase annotation contains free Cys220. **(B)** The region from 346 to 406 in the garlic alliinase annotation contains free Cys350 and the disulfide bridge Cys368–Cys376. Thiols corresponding to Cys220 can be substituted in some cases by serine, whereas all other cysteine residues are strongly conserved. Cysteines are shown with a light grey background. All amino-acid residues that incorporate transmission of interactions from an S-S bridge to PLP are conserved in all alliinases. They are highlighted in dark grey. Sources and genes: AAB32477, *Allium sativum* (garlic); AAK95657, *Allium chinense* (Chinese onion); AAK95660, *Allium schoenoprasum* (chives); AAA32639, *Allium ascalonicum* (shallots); AAK95656, *Allium wakegi* (bunching onion); AAA92463, *Allium cepa* (onion); AAF36437, *Allium cepa* (onion roots); AAK95662, *Allium sativum* (garlic roots); BAA20358, *Allium tuberosum* (Chinese chives); AAD51703, *Allium giganteum* (giant onion).

Figure 8. Free thiols of alliinase. **(A)** Relative locations of Cys220 and Cys350 (green) on the surface of the alliinase monomer. **(B)** Respective orientations of Cys220 and Cys350 relative to A. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

alliinase, as well as the relatively long distances from modified free cysteines to the active center of the enzyme (see Fig. 5). Using the experimentally obtained d_1/d value in the EPR spectrum of the double-labeled enzyme at 120 K (see Fig. 3) and calibration curves describing d_1/d dependence on the distance between

Figure 9. Solid-phase NTB assay of biotin-alliinase using streptavidin-coated plates. Streptavidin-coated plates were prepared as described in Methods. A solution of biotin-alliinase in PBS (0.5 mg/mL) or unmodified alliinase in PBS (0.5 mg/mL) as control was incubated in the wells for 2 h at room temperature. Binding of biotinylated alliinase to streptavidin was estimated by assay of its activity using the colorimetric NTB method and 10 mM alliin as a substrate. The reaction was followed by an ELISA Reader at various intervals at 405 nm.

two radicals,²⁷ we found that the distance between the labeled cysteines, Cys220 and Cys350 was >22 Å. This result is in good agreement with the known alliinase structures (4, 6). The closest distance (see Fig. 5) between the two free cysteines Cys220 and Cys350 in the same monomeric subunit is 28 Å. Note that the distance between the two Cys 220 residues in the different subunits of the dimer is 48 Å (see Fig. 5). Although the Ellman's reagent, DTNB, did not react with the native enzyme, ³H-allicin was shown to modify 1.4 —SH groups of alliinase.³¹ This was not surprising, as allicin is a small hydrophobic molecule that can efficiently modify —SH groups, even those deeply buried in biological membranes or in the protein core.^{14,32} We found that the cysteine residues Cys368 (located in the β -strand 363–368) and Cys376 (located in a short α -helix 376–383) form a disulfide bridge that locks the 368–378 Ω loop in its narrow part. This tight loop locks the structure between two subdomains of the C-terminal part of domain 1 and flanks a series of interactions along two short antiparallel β -strands, 399–403 and 363–368, with PLP on the opposite flank (see Fig. 6). The Cys368–Cys376 bridge helps to reinforce the interface between domains 1 and 2 and, therefore, plays an important role in maintaining the geometry of the active site, which comprises residues from both domains. All cysteine residues in alliinases from different strains of the genus *Allium* have been found to be highly conserved with the exception of a free thiol cysteine (Cys220 in garlic alliinase) (see Fig. 7) that is sometimes substituted by serine.^{33,34}

Recent years have seen significant progress in the study of the biological activity and application of sulfur-containing compounds from garlic. It is now accepted that allicin a product of the alliinase reaction is a parent substance for most of those compounds. Therefore, studies on the enzyme structure and regulation of allicin production seem to be of crucial importance in all fields of garlic thiosulfinate generation and application. A recent development in the application of alliinase is the approach to in situ production of allicin from its precursor (alliin) by using targeted alliinase for the treatment of cancer.^{35,36} In view of our finding that the modification of free thiols of alliinase does not affect the enzyme activity, its conjugation via —SH groups looks rather promising. In the present study we used this approach to prepare alliinase modified with biotin. The conjugates demonstrated unchanged activity compared with intact enzyme, and were capable of interacting with streptavidin (see Fig. 9).

In conclusion, as follows from sequence analysis all S—S bridges in the alliinase molecule are strongly conserved and probably play an important role in maintenance of the properly folded active state. The presence of two free —SH groups, belonging to Cys220 and Cys350, in each subunit of alliinase from garlic was demonstrated in this study. The free thiols are located far from the active site of the enzyme, and

their modification does not affect the enzyme's activity. The possibility of preparing active conjugated alliinase using the —SH reagent biotinyl maleimide was demonstrated. b-S-Alliinase interacted with streptavidin, indicating that the conjugated enzyme can be used for alliinase targeting.

Methods

Materials *N*-(4-dimethylamino-3,5-dinitrophenyl) maleimide (DDPM) was purchased from Aldrich. Biotin-maleimide, TPCK-treated trypsin, chymotrypsin, pepsin, dithiothreitol (DTT), 5,5'-dithiobis-(2-nitrobenzoic acid) Ellman's reagent, DTNB, 4,4'-dithiodipyridine (DTP), 2-iodoacetic acid, PLP, and guanidine-HCl were purchased from Sigma (St. Louis). The stable nitroxide biradical, bis (1-oxy-2,2,5,5-tetramethyl-3-imidazo-line-4-yl) disulfide, was synthesized as described in Ref. ³⁷, and stock solutions of this reagent (0.5 mM in ethanol) were stored at –20°C. The reagent 2-nitro-5-thiobenzoate (NTB) was prepared as described.³⁸ Alliinase (specific activity 380 U/mg) was isolated from garlic as described previously.^{5,39} Protein was concentrated with Amicon® Centricon® YM-30 Centrifugal filter devices (Millipore).

Activity assay

Alliinase activity was assayed using the NTB method, which is based on the reaction of enzymatically produced allicin with NTB.⁴⁰ The standard reaction mixture contained NTB (10^{-4} M) in 50 mM sodium phosphate buffer pH 6.5, EDTA (1 mM), PLP (2×10^{-5} M), and alliinase ($2.5\text{--}10 \times 10^{-3}$ units). The enzymatic reaction at 23°C was started by the addition of alliin (10 mM), and the initial rate of reaction was monitored spectrophotometrically by recording of the decrease in absorbance at 412 nm. The total volume of the reaction mixture was 1.0 mL. To determine enzyme activity, the amount of NTB consumed in the reaction with allicin was calculated according to the formula: $\epsilon_{412} = 14,150 \text{ M}^{-1} \text{ cm}^{-1}$ (1 mol NTB is equivalent to 0.5 mol of allicin; thus, this corresponds to 1 mol of pyruvic acid). Protein concentration was measured at 280 nm, using $\epsilon_{280} = 77,000 \text{ M}^{-1} \text{ cm}^{-1}$ (ϵ_{280} 0.1% = 1.54) for alliinase.²

Titration of alliinase-free thiols and preparation of an S-modified alliinase

To determine free thiols in the alliinase molecule, alliinase-free thiols were titrated at room temperature using DTNB and DTP. All titrations were performed with 20-fold excess of reagent, corresponding to concentrations of 1.0–10.0 mM. To prepare —SH-modified alliinase, 10 mg of the enzyme in 2.0 mL of phosphate-buffered saline (PBS) containing 10% glycerol was treated with 50 μ L of 0.2M DTP in DMSO for 3 h at room temperature. The modified alliinase preparation was gel-filtered on a Sephadex G-25 column,

concentrated to 5 mg/mL with Centricon YM-30, and kept at -20°C in 50% glycerol.

Isolation and sequencing of free thiol-containing peptides from alliinase

To isolate free thiol-containing peptides, alliinase (1 mg in 0.25 mL of PBS containing 10% glycerol and 2.5 mM EDTA) was treated with 10 μL of 40 mM DDPM in DMSO for 1 h at room temperature, and then extensively dialyzed against PBS. The protein was denatured by the addition of solid guanidine-HCl to 6M and DTT to 6 mM. The solution was left overnight and then 6 mg of 2-iodoacetic acid was added in 0.5N NaOH for S-carboxymethylation (30 min in the dark). The reaction was stopped by the addition of excess of DTT followed by exhaustive dialysis against water. The resulting pellet of alkylated protein was collected by centrifugation and suspended in 0.2 mL of 50 mM ammonium bicarbonate. It was then digested with trypsin or chymotrypsin, or suspended in 0.01N HCl, adjusted to pH 2.0, and digested with pepsin. The ratio of substrate to enzyme was 25:1. Digestion was performed at 37°C overnight in the case of trypsin or chymotrypsin and for 2 h in the case of pepsin. Reactions were stopped by acidification with trifluoroacetic acid, and the dimethylamino-dinitrophenyl-S-peptides that were formed were isolated by reversed-phase HPLC. For sequencing, peptides were separated in a Vydac Protein & Peptide C-18 column (4.6 mm $\times$ 250 mm) using the HPLC system of Jasco, equipped with a Jasco UV-1570 OM detector. Peptides were eluted from the columns by an acetonitrile gradient of 0–80% in 0.1% aqueous trifluoroacetic acid at a flow rate of 0.2 mL/min.

Chemical modification of alliinase with nitroxyl biradical

Reaction was initiated by mixing of 50 μL of an aqueous solution of the nitroxyl biradical (20 μM , pH 7.3) with an equal volume of alliinase (4 μM) in a mixer placed inside an EPR spectrometer resonator (mixing time, 15 s). The kinetics of the thiol–disulfide exchange between the nitroxyl biradical and the $-\text{SH}$ groups of alliinase were monitored by following the appearance of the monoradical component in the EPR spectrum.^{25,41} To chemically modify the reactive cysteine residues, alliinase (100 nmol in 1 mL) was incubated with a 50-fold excess of nitroxyl biradical for 5 h at room temperature. Excess reagent was removed by gel filtration on a Sephadex G-25 column. Fractions corresponding to the chemically modified protein were combined and concentrated to a volume of 0.4 mL by ultrafiltration using Centricon YM-30.

S-biotinylation of alliinase

Alliinase 10 mg in 2.0 mL of PBS containing 10% glycerol) was treated by slow addition of 20 μL of 0.2M biotin-maleimide in DMSO (1 h, room temperature).

The resulting conjugate, b-S-alliinase was gel-filtered on a Sephadex G-25 column, concentrated to 5 mg/mL with Centricon YM-30, and kept at -20°C in 50% glycerol.

Measurement of solid-phase activity of b-S-alliinase

Streptavidin (10 $\mu\text{g}/\text{mL}$ water) was incubated in 96-well Nunc-ImmunoTM plates overnight at 4°C . Unbound protein was removed by five washes with PBS/0.05% Tween-20. Nonspecific sites in the wells were blocked by incubation for 2 h in PBS containing 1 mg/mL of bovine serum albumin. Unbound bovine serum albumin was removed by three washes with PBS/0.05% Tween-20. A solution of b-S-alliinase in PBS (0.5 mg/mL) or unmodified alliinase in PBS (0.5 mg/mL) as a control was incubated in the wells for 2 h at room temperature. After removal of unbound proteins by extensive washing with PBS/0.05% Tween-20, a solution of NTB reagent ($\text{OD}_{412} \sim 0.5\text{--}0.6$) containing 10 mM alliin was added. The reaction was quantified at various intervals by an ELISA reader at 415 nm. All ELISA experiments were performed at least twice.

Analytical procedures

Low-temperature EPR experiments were carried out in Hepes/glycerol (50/50) solutions. The concentration of the nitroxyl radical covalently bound to alliinase was determined by double integration of the EPR spectra, using the stable nitroxyl radical 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) for calibration. A temperature unit control (Euroterm ER 3141VT, Bruker) was used for measurements at 120 K with accuracy of ± 1 K. EPR spectra were measured on a Bruker ELEXSYS 500 spectrometer (X band) in a 60- μL flat cell (at room temperature) or in capillary (120 K). Absorption spectra were measured with a Hewlett-Packard 8452A diode array spectrophotometer. Circular dichroism (CD) spectra were recorded by an Aviv Model 202 CD spectrometer, as described.⁴² Data are expressed as the mean residue ellipticity $[\theta]$ [(deg cm^2)/dmol]. N-terminal peptide sequences were determined with a model 491 Procise protein sequencer (Applied Biosystems).

Structural data

Structural analysis of the cysteine residues in alliinase is based on the previously reported X-ray crystal structure,⁶ PDB accession code 2HOX. The programs Coot⁴³ and Pymol⁴⁴ were used for geometric calculations and structural representations, respectively.

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