Ajoene Is an Inhibitor and Subversive Substrate of Human Glutathione Reductase and *Trypanosoma cruzi* Trypanothione Reductase: Crystallographic, Kinetic, and Spectroscopic Studies

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Ajoene ((*E*,*Z*)-4,5,9-trithiadodeca-1,6,11-triene 9-oxide), a garlic-derived natural compound, is a covalent inhibitor as well as a substrate of human glutathione reductase (GR) and *Trypanosoma cruzi* trypanothione reductase (TR). The 2.1-Å resolution crystal structure of GR inhibited by (*E*)-ajoene revealed a mixed disulfide between the active site Cys58 and the $CH_2{=}CH{-}CH_2{-}SO{-}CH_2{-}CH{=}CH{-}S$ moiety of ajoene. The modified enzyme has a markedly increased oxidase activity when compared to free GR. GR reduces (*Z*)-ajoene with a k_{cat}/K_m of 6.8×10^3 M^{-1} s^{-1} yielding 4,5,9-trithiadodeca-1,6,11-triene (deoxyajoene) and 4,8,9,13-tetrathiahexadeca-1,6,10,15-tetraene as stable reaction products. The reaction leads also to the formation of single-electron reduced products and concomitantly superoxide anion radicals as shown by coupling the reaction to the reduction of cytochrome *c*. The interactions between the flavoenzymes and ajoene are expected to increase the oxidative stress of the respective cell. The antiparasitic and cytostatic actions of ajoene may at least in part be due to the multiple effects on key enzymes of the antioxidant thiol metabolism.

Introduction

Garlic (*Allium sativum*) has been used for more than 400 years in traditional medicine against cardiovascular diseases, cancer, and infections. A major sulfur component of fresh garlic is alliin (*S*-2-propenyl-L-cysteine *S*-oxide) which is converted into allicin (diallyl thiosulfinate) upon maceration of the bulb. Allicin, the antibacterial component of garlic, is spontaneously degraded, a major product being (*E*,*Z*)-ajoene (4,5,9-trithiadodeca-1,6,11-triene 9-oxide).[1] Ajoene is well-known for its inhibitory effect on platelet aggregation[2] and its cytotoxic,[3] antifungal,[1] antiviral,[4] antitrypanosomal,[5] and antimalarial activity.[6] On the biochemical level, it is an inhibitor of cholesterol synthesis,[7] 5-lipoxygenase, and prostaglandin synthase[8] and has been discussed to interfere with the thiol homeostasis of the cell,[9] but the molecular mechanisms of these reactions have not been revealed. Recently ajoene has been reported to induce apoptosis in human leukemic cells, and the process has been suggested to occur via peroxide production and activation of the transcription factor NF-κB.[10]

Diallyl trisulfide (DATS), another transformation product of allicin, has been used since 1981 in China for the treatment of bacterial, fungal, and parasitic infections in humans and has in vitro activity against several *Trypanosoma* species.[11]

Glutathione reductase (GR, EC 1.6.4.2) is a key enzyme in the cell's defense mechanisms against oxidative stress. The flavoprotein is responsible for maintaining the reducing intracellular milieu by catalyzing the reaction: $NADPH + GSSG + H^+ \rightarrow NADP^+ + 2GSH$.[12,13] Trypanothione reductase (TR, EC 1.6.4.8) occurs in the causative agents of African sleeping sickness (*Trypanosoma brucei gambiense*, *T. b. rhodesiense*), Nagana cattle disease (*T. congolense*), Chagas' disease (*T. cruzi*), and the three manifestations of leishmaniasis.[14] In these parasitic protozoa TR replaces GR. TR catalyzes the reduction of trypanothione disulfide (TS_2) to trypanothione [$T(SH)_2$, N^1,N^8-bis(glutathionyl)spermidine]: $NADPH + TS_2 + H^+ \rightarrow T(SH)_2 + NADP^+$.

Mechanistically and structurally TR and GR are closely related.[12,13,15] Both enzymes occur in two stable forms, E and EH_2. E is characterized by an active site disulfide bridge between Cys58 and Cys63 (in human GR; Cys52 and Cys57 in *T. cruzi* TR). The two-electron-reduced EH_2 form contains the two cysteines as a dithiol.[12,16] In the EH_2 state, Cys63 (in GR) forms the characteristic charge-transfer complex with the prosthetic group FAD, and Cys58 reacts with glutathione disulfide to a mixed disulfide as reaction intermediate. Cys58 (Cys52 of TR) is highly reactive toward alkylating or carbamoylating compounds.[17]

Both FAD disulfide oxidoreductases have only minute intrinsic oxidase activities (oxidation of NADPH in the absence of the disulfide substrate), but several com-

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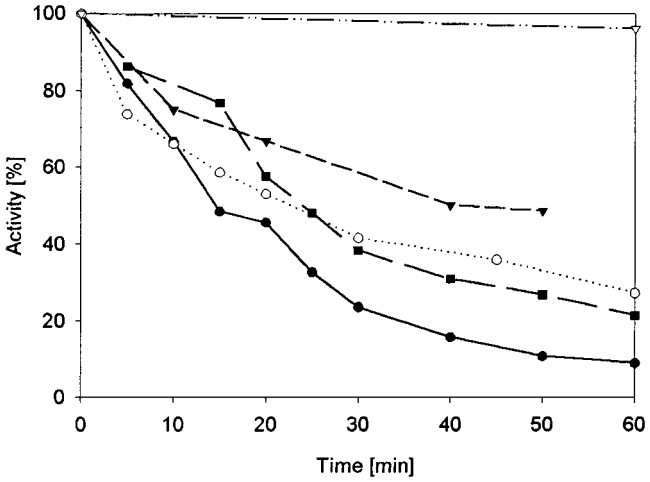


Figure 1. Inhibition of human glutathione reductase by ajoene. In a total volume of 200 μL, 0.6 U GR was incubated with 200 μM NADPH and 12.5 μM (▼), 50 μM (■), and 200 μM (●) (*E*)-ajoene and 200 μM (*Z*)-ajoene (○), respectively, in GR assay buffer, pH 6.9, at 37 °C. The control sample (▽) contained NADPH and enzyme but no ajoene. After the indicated time intervals, 2-μL aliquots were removed and assayed for remaining activity in a standard GR assay. The values measured at $t = 0$ and 60 min are the mean of two measurements which differed by less than 5%. The other values are single determinations because the narrow time frame did not allow duplicate measurements. The complete data set was measured three times.

pounds such as the herbicide paraquat (methyl viologen) or nitrofuran derivatives strongly induce this side reaction. The one-electron reduction of such turncoat inhibitors (subversive substrates) leads to the formation of reactive oxygen species. The concomitant increase of the cellular oxidative stress is an attractive mechanism for antiparasitic and antitumor drugs.[13]

Here we report on the interactions of ajoene and diallyl trisulfide with human glutathione reductase and *T. cruzi* trypanothione reductase. Kinetic, crystallographic, and spectroscopic studies reveal the molecular mode of inhibition and the products formed in the enzymic reduction of ajoene.

Results

Inhibition of Human GR and *T. cruzi* TR by Ajoene. Ajoene is a covalent inhibitor of human glutathione reductase and *T. cruzi* trypanothione reductase. The reaction is time- and temperature-dependent. Both enzymes react exclusively in the two-electron-reduced state (EH_2) in which the two active site cysteines form a dithiol.

Incubation of 0.3 μM GR with 200 μM (*E*)-ajoene in the presence of NADPH leads to 50% inhibition within 15 min at 37 °C (Figure 1). Under identical conditions the (*Z*)-isomer reacts slightly slower which is probably due to the much faster consumption of (*Z*)-ajoene in the competing enzymic reduction of the compound (see below). No inhibition of the enzyme by ajoene is observed in the absence of NADPH, which is indicative of an active site cysteinyl residue being altered. The modification of the enzyme is stable upon extensive dialysis but can be reversed in the presence of a strong reductant. After a 4-h incubation with 4 mM dithiotreitol, but not glutathione, 70% of the initial GR activity is regained. In contrast, 5 mM GSH, but not DTT, in the reaction mixture largely interferes with inhibition. This specific protection may be due to formation of a mixed disulfide between Cys58 and glutathione which has been observed as an intermediate in the reaction of yeast glutathione reductase with glutathione.[18] Even if under in vivo conditions millimolar concentrations of GSH may slow the reaction, the equilibrium will shift toward the modified protein species since the alteration is not abolished by glutathione. Ajoene-modified GR is unable to reduce GSSG but shows a markedly increased oxidase activity. Substrate-independent oxidation of NADPH increases by a factor of about 5 in the ajoene-inactivated enzyme, and the rate of cytochrome *c* reduction is 10-fold higher when compared to the native enzyme. Modification of Cys58 is expected to shift electron density toward the flavin ring and thus to promote reduction of molecular oxygen which is supposed to occur directly at the isoalloxazine ring.

Trypanothione reductase is also inhibited by ajoene. Incubation of *T. cruzi* TR with 200 μM (*E*)- or (*Z*)-ajoene at 37 °C in the presence of 200 μM NADPH leads to 50% and 40% inhibition, respectively, within 60 min. The presence of 1 mM trypanothione in the reaction mixture practically prevents inhibition. In contrast, 1 mM DTE only slows down the rate of reaction.

Modification of GR and TR by (*E*)-ajoene causes a decrease and slight hypsochromic shift of $\lambda_{max,visible}$ of the flavin spectrum and a concomitant absorbance increase around 530 nm. This broad band is characteristic for the charge-transfer complex formed between Cys63 (in GR; Cys 57 in TR) and the flavin in the reduced enzyme species[12] and is strong evidence for Cys58 (in GR; Cys 52 in TR) being the altered protein residue. In both modified proteins the charge-transfer absorption is lower than in the respective EH_2 species.[19] This finding fits nicely the structural data of ajoene-modified GR (see the next section) where Cys63 occupies two alternative conformations of which only one should be able to undergo the charge-transfer interaction with the flavin ring.

X-ray Diffraction Analysis of GR Covalently Modified by (*E*)-Ajoene. The three-dimensional structure of human glutathione reductase covalently modified by ajoene was determined to 2.1-Å resolution. The statistics of the diffraction and refinement data are summarized in Table 1. The refined model consists of one protein monomer with 3499 non-hydrogen atoms, one FAD (53 atoms), one inhibitor moiety (9 atoms), one chloride ion at the surface of the protein, and 144 solvent molecules (Figures 2 and 3). Three probable ajoene fragments were incorporated in the protein model (see Table 1). The shape of the electron density for the inhibitor is consistent with $CH_2{=}CH{-}CH_2{-}SO{-}CH_2{-}CH{=}CH{-}S$ (fragment 2) attached to Cys58 of the protein (Figure 3A). The electron density for the sulfur atoms forming the disulfide bridge and the sulfoxo group, respectively, is clearly visible in the $(F_{obs} - F_{calc})$ exp $i\alpha_{calc}$ and $(2F_{obs} - F_{calc})$ exp $i\alpha_{calc}$ maps. The shape and intensity of the electron density could not be explained by a thiosulfinate (fragment 3) or by an *S*-allyl moiety (fragment 1) at Cys58. Only after incorporation of fragment 2 attached to Cys58 in the model did the

Table 1. Data Collection and Refinement Statistics

parameter				
space group[a]	$P2_1$		$C222_1$	
	overall (∞−2.2 Å)	outer shell (2.3−2.2 Å)	overall (∞−2.1 Å)	outer shell (2.3−2.1 Å)
no. of measured reflns	153865	9595	147210	16603
no. of unique reflns	56446	6096	34707	7571
R_{mrgd} (%)[b]	10.3	22.6	11.7	30.3
completeness (%)	93.2	80.3	96.4	88.9
I/σ	13.9	2.6	9.3	1.5
	Refinement in $C222_1$			
	fragment 1 $CH_2{=}CH{-}CH_2{-}S{-}$	fragment 2 $CH_2{=}CH{-}CH_2{-}S(O){-}CH_2{-}CH{=}CH{-}S{-}$	fragment 3 $CH_2{=}CH{-}CH_2{-}S(O){-}$	
resolution (Å)	20−2.1	20−2.1	20−2.1	
R_{cryst} (%)[c]	18.2	18.0	18.1	
R_{free} (%)[d]	23.3	23.0	23.4	
rms bond distance deviation (Å)	0.010	0.009	0.010	
rms bond angle deviation (deg)	0.975	0.925	0.984	
mean *B*-factor of the protein atoms (Å^2)	22.6	22.5	22.6	
mean *B*-factor of the solvent molecules (Å^2)	24.4	24.2	24.5	
mean *B*-factor of the inhibitor atoms (Å^2)	36.2	46.1	41.5	

[a] For space group discussion, see Experimental Procedures. [b] $R_{mrgd} = \sum_h \sum_i |I_{hi} - I_h| / \sum_{hi} I_{hi}$, where h is unique reflection indices and I_{hi} is the intensity of symmetry equivalent reflections giving a mean value of I_h. [c] $R_{cryst} = \sum |F_{obs} - F_{model}| / \sum F_{obs}$, where F_{obs} and F_{model}, are observed and atomic model structure factor amplitudes, respectively. [d] *R*-factor calculated for 7% of randomly chosen reflections which were excluded from the refinement.

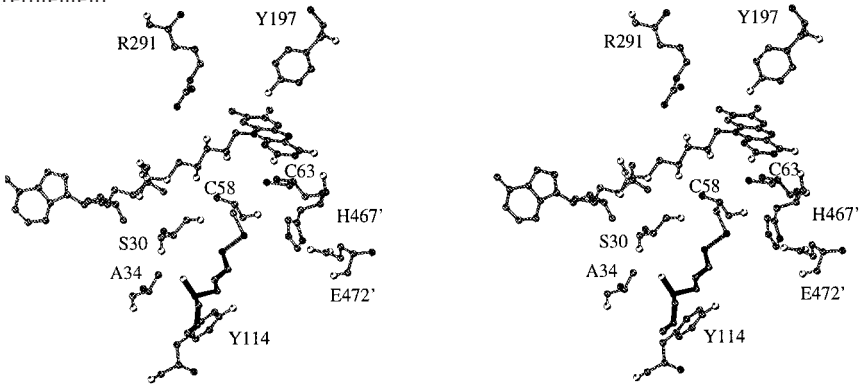


Figure 2. Stereoplot of the active site of human GR with the $CH_2{=}CH{-}CH_2{-}SO{-}CH_2{-}CH{=}CH{-}S{-}$ moiety of ajoene covalently bound to Cys58. The isoalloxazine ring of FAD forms the center of the active site. The NADPH binding site on the *re*-face of the flavin is represented by Tyr197. Cys58 and Cys63 form the redox active dithiol/disulfide. His467' and Glu472' which are also involved in catalysis are contributed by the other subunit. Sulfur atoms are depicted by filled circles. The dashed line represents the disulfide bridge formed between the inhibitor fragment (bold lines) and Cys58. The hydroxyl group of Ser30 is located 3.6 Å from the sulfur atom of the ajoene fragment which forms the disulfide bond with Cys58. The NH group of Ala34 is 3.5 Å from the sulfoxo oxygen. Figure was prepared using BOBSCRIPT.[20]

positive ($F_{obs} - F_{calc}$) exp iα_{calc} electron density disappear and the ($2F_{obs} - F_{calc}$) exp iα_{calc} electron density map improve upon refinement, especially for the terminal carbon atoms of the inhibitor fragment. The free *R*-factor was slightly lower than in the refinements with the other two fragments (Table 1). Refinement of individual temperature factors revealed values of about 40 Å^2 for the first four atoms of the bound ajoene fragment. The higher temperature factors of about 47 Å^2 for the terminal atoms reflect the mobility of the distal allyl group which is not fixed by contacts with the protein. The refined occupancy of the inhibitor indicates that the degree of modification is at least 75% in agreement with the biochemical data. The residual protein seems to be present in the oxidized form since there is some density corresponding to the disulfide bridge between Cys58 and Cys63 (Figure 3A).

In the ajoene-inhibited GR the ligand forms two direct interactions with the protein. The sulfur atom of the ajoene moiety forming the disulfide bridge with Cys58 is within hydrogen-bonding distance to the hydroxyl group of serine 30 (3.6 Å). Another direct contact with the protein may be to the main chain NH group of Ala34 which is in 3.5-Å distance from the sulfoxo group of the ajoene fragment.

The ($F_{obs} - F_{calc}$) exp iα_{calc} map indicates that Cys63 has two alternative conformations. The main location coincides with that in E and EH_2 (which differ by only about 0.1 Å) as well as several enzyme species with covalently modified Cys58.[16,17,21−23] A refinement cycle including both conformations for Cys63 resulted in a model that fits well the ($F_{obs} - F_{calc}$) exp iα_{calc} and ($2F_{obs} - F_{calc}$) exp iα_{calc} electron density maps (Figure 3). In the alternate conformation Cys63 does not seem to form a disulfide bridge with Cys58 since the distance of 5.5 Å is too long and negative difference density appeared between Cys58 and Cys63 if the disulfide bridge was built with Cys63 in this conformation. In contrast, the disulfide bridge built with the conventional conformation of Cys63 fits the electron density maps well.

Reduction of Ajoene by Human GR and *T. cruzi* TR. The reduction of ajoene by human GR was followed at 340 nm by measuring NADPH oxidation which records single- and two-electron processes and at 550

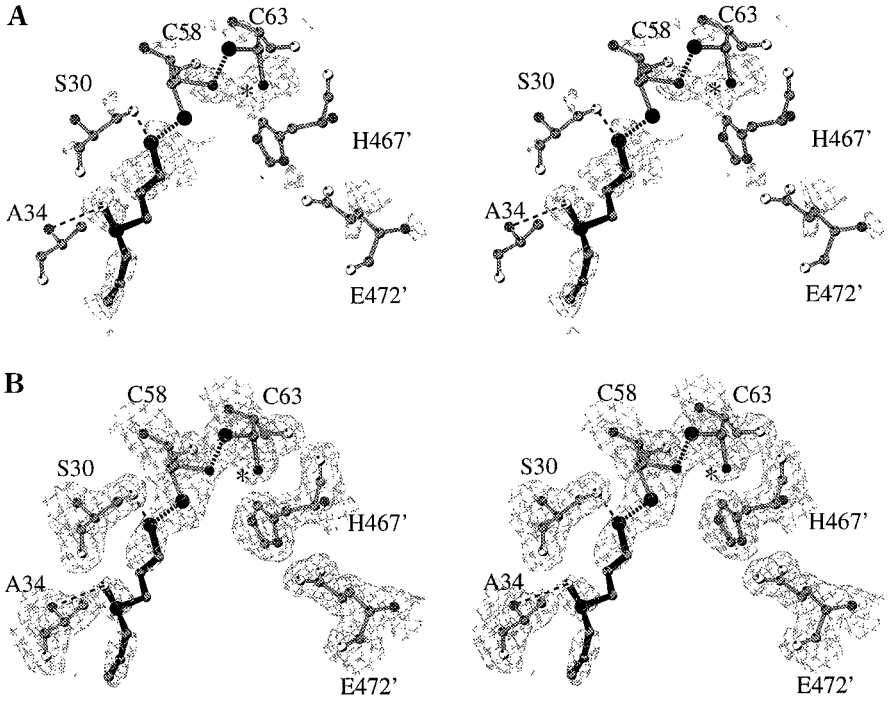


Figure 3. Active site of GR modified by the ajoene fragment, same view as in Figure 2. (A) Initial difference Fourier map calculated with coefficients ($F_{obs} - F_{calc}$) exp iα_{calc} that contained the protein coordinates of the refined model with Cys58 and Cys63 in their main conformation but no coordinates for the inhibitor. The inhibitor fragment (bold lines) is covalently bound to Cys58 via a disulfide bridge (coarse dashed line). Potential hydrogen bonds with the OH group of Ser30 and the main chain NH group of Ala 34 are indicated by fine dashed lines. In the ajoene-modified enzyme, Cys58 and Cys63 each occupy two different positions. The major position is shown with enlarged atoms for S_γ. The additional electron density for Cys58 corresponds to the position of the residue in the oxidized enzyme and reveals that some of the protein is not modified but forms the disulfide bridge with Cys63 (coarse dashed line). The major conformation of Cys63 is that usually observed in the oxidized and reduced enzyme as well as in several protein species modified at Cys58.[16,17,21−23] In the ajoene-modified GR, Cys63 shows an alternative conformation (marked by an asterisk; for details see text). The contour level is 2.1σ. (B) Refined model containing the coordinates for both conformations of Cys58 and Cys63, respectively, and of the inhibitor fragment with the final ($2F_{obs} - F_{calc}$) exp iα_{calc} electron density map. The contour level is 1.1σ. Figure was prepared using BOBSCRIPT.[20]

nm by the reduction of cytochrome *c* which is a marker for single-electron reductions (Table 2). In the latter case the following reactions are thought to occur:

$$2X + NADPH + H^+ \rightarrow 2X^\bullet + NADP^+ + 2H^+ \quad (1)$$

$$2X^\bullet + 2 \text{ cytochrome } c\ (Fe^{3+}) \rightarrow 2X + 2 \text{ cytochrome } c\ (Fe^{2+}) \quad (2)$$

$$2X^\bullet + 2O_2 \rightarrow 2O_2^{-\bullet} + 2X \quad (3a)$$

$$2O_2^{-\bullet} + 2 \text{ cytochrome } c\ (Fe^{3+}) \rightarrow 2O_2 + 2 \text{ cytochrome } c\ (Fe^{2+}) \quad (3b)$$

Compound X is reduced to a single-electron-reduced intermediate that transfers the electron either directly (reaction 2) or via molecular oxygen onto cytochrome *c* (reaction 3a,b). The involvement of the $O_2/O_2^{-\bullet}$ couple in the reaction can be demonstrated by inhibiting the reduction of cytochrome *c* (reaction 3b) with superoxide dismutase (SOD).

GR reduces (*Z*)-ajoene with a k_{cat}/K_m of 6.8×10^3 M^{-1} s^{-1}. Reduction of the (*E*)-isomer is nearly 10 times slower when following the oxidation of NADPH, but the rates for cytochrome *c* reduction are comparable for both isomers. In the presence of ajoene the rate of cytochrome *c* reduction increases by 1−2 orders of magnitude which clearly shows that reduction of ajoene by GR generates single-electron-reduced products and superoxide anion radicals.

Since millimolar concentrations of glutathione interfere with the covalent modification of GR by ajoene (see above), the influence of the thiol on the reduction of ajoene by the enzyme was studied. As summarized in Table 2, GSH does not inhibit the reaction. The even slightly higher rates in the presence of GSH may be for several reasons: for instance, activation of the enzyme by the thiol and formation of the natural substrate GSSG via an ajoene-mediated oxidation of GSH.[3]

Ajoene is also a substrate of trypanothione reductase; the K_m values for both isomers are ≥2 mM. The parasite enzyme reduces (*E*)- and (*Z*)-ajoene with a k_{cat}/K_m of 3.4 $\times 10^2$ M^{-1} s^{-1} (Table 2).

Analysis of the Reaction Products of the GR-Catalyzed Reduction of Ajoene. (*Z*)-Ajoene was incubated with human GR in the presence of NADPH, and the reaction products were separated by HPLC as described under Experimental Procedures. A first indication that quite hydrophobic products are formed was the developing turbidity of the reaction mixture. HPLC analysis revealed six major peaks. The first two peaks at 1.1 and 2.7 min contained NADPH together with other hydrophilic compounds and unreacted ajoene, respectively. The two most prominent peaks at 20 and 43 min were subjected to ^1H NMR analysis. Although the NMR spectra are complex (not shown), the com-

Table 2. Kinetic Data for the Reduction of Ajoene and DATS by Human Glutathione Reductase and *T. cruzi* Trypanothione Reductase[a]

	oxidation of NADPH			reduction of cytochrome *c*
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1})
		Glutathione Reductase		
(*Z*)-ajoene	2.6 ± 0.3	380 ± 50	6840 ± 900	0.06 ± 0.02
(*E*)-ajoene	0.34 ± 0.03	800 ± 100	425 ± 60	0.1 ± 0.01
(*E*)-ajoene + GSH	1.47 ± 0.2			0.32 ± 0.04
DATS	0.16 ± 0.02	160 ± 30	1000 ± 300	0.21 ± 0.04
control	0.006 ± 0.003			0.0011 ± 0.0002
control + GSH	0.016 ± 0.003			nd
		Trypanothione Reductase		
(*Z*)-ajoene	0.67 ± 0.06	≥2000	335 ± 30	nd
(*E*)-ajoene	0.67 ± 0.06	≥2000	335 ± 30	nd
control	0.028 ± 0.005			nd

[a] The kinetics of GR were measured at 25 °C in GR assay buffer, pH 6.9, at an NADPH concentration of 150 μM. The kinetics of TR were measured at 25 °C in TR asssay buffer, pH 7.5, as described under Experimental Procedures. The control assays which contained only NADPH and the enzyme represent the intrinsic oxidase activity of the enzymes. Oxidation of NADPH was followed at 340 nm. Reduction of cytochrome *c* was measured at 550 nm (ϵ = 19.1 mM^{-1} cm^{-1}). The effect of glutathione on the reaction rates of GR was studied by adding 1 mM GSH. Addition of 5 μg of SOD decreased the rate of cytochrome *c* reduction in all reactions by about 25%. nd, not determined, because of the strong cytochrome *c* reductase activity of TR.[24] The values are the mean of at least three independent measurements.

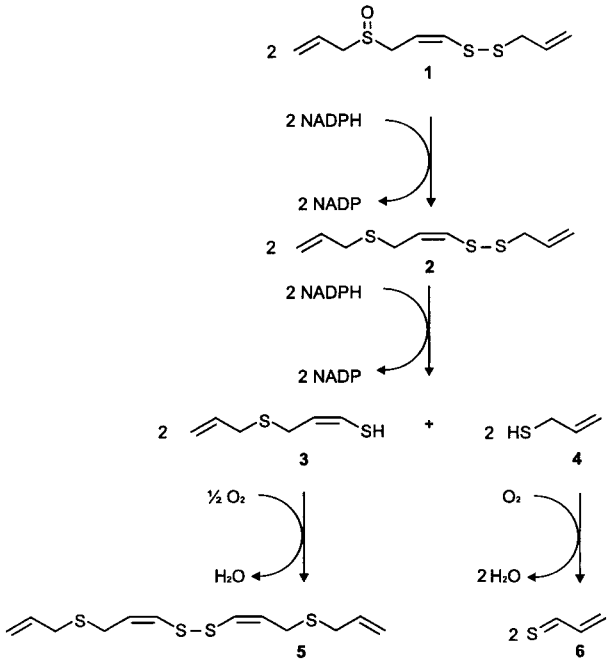


Figure 4. Hypothetical reaction scheme for the reduction of ajoene by human glutathione reductase. The NADPH-dependent reduction of (*Z*)-ajoene (**1**) yielded several reaction products which were separated by HPLC. Two stable products were characterized by NMR spectroscopy as 4,5,9-trithiadodeca-1,6,11-triene (deoxyajoene, **2**) and 4,8,9,13-tetrathiahexadeca-1,6,10,15-tetraene (**5**). The turbidity and pungent smell that developed in the progress of the reaction are evidence for the formation of allylmercaptan (**4**) which spontaneously oxidizes to the volatile thioacroleine (**6**).

pounds could be identified since their spectra were identical with those of the reaction products between ajoene and cysteine.[25] The compound with a retention time of 20 min is 4,5,9-trithiadodeca-1,6,11-triene (compound **2** in Figure 4), and the one with a retention time of 43 min is 4,8,9,13-tetrathiahexadeca-1,6,10,15-tetraene (compound **5**). Compound **2** can be designated as deoxyajoene since it represents ajoene in which only the sulfoxo group is reduced. The symmetrical compound **5** contains twice the allyl−S(O)−allyl−S moiety of ajoene in which the sulfoxo group had been reduced. The strong smell of the reaction mixture indicated formation of the volatile allylmercaptan (compound **4** in Figure 4) which rapidly reacts with oxygen to form thioacrolein. With Ellman's reagent free thiols could not be detected in accordance with the rapid formation of oxidized products.

Reduction of Diallyl Trisulfide (DATS) by Human GR. DATS (CH_2=CH−CH_2−S−S−S−CH_2−CH=CH_2), another degradation product of alliin, is also a substrate of GR (Table 2) but, in contrast to ajoene, does not inhibit the enzyme. GR catalyzes the reduction of DATS with a k_{cat}/K_m of 1000 M^{-1} s^{-1}. DATS causes a cytochrome *c* reduction rate of GR which is higher than that with ajoene. In addition, NADPH oxidation and cytochrome *c* reduction occur at similar rates which shows that single-electron-reduced species are the main reaction products. The nature of the single-electron-reduced products is not known. For diallyl disulfide the dissociation energy of the C−S bond has been reported to be much lower than that of the S−S bond favoring C−S homolysis.[1,26] In diallyl trisulfide the estimated S−S bond energy is very similar to the C−S bond energy so that both S−S and C−S homolysis should occur.[26]

Discussion

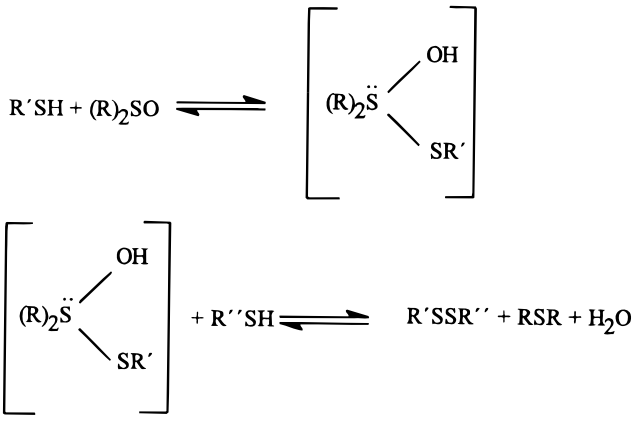
Several thiol-containing enzymes such as gastric lipase,[27] prostaglandin synthase, lipoxygenase,[8] xanthine oxidase, and glyceraldehyde-3-phosphate dehydrogenase[25] have been reported to be inhibited by ajoene, but the molecular mechanism of inhibition has not yet been elucidated. As shown here, ajoene is a covalent inhibitor of human glutathione reductase (GR) and *T. cruzi* trypanothione reductase (TR). The crystallographic analysis of GR inhibited by ajoene revealed the presence of an allyl−S(O)−allyl−S−group at Cys58 (Figures 2 and 3). The inhibitor moiety corresponds to the larger fragment formed upon reduction of the ajoene disulfide. The overall orientation of the inhibitor in the active site is similar to that of CysI and GlyI of glutathione in the mixed disulfide between GSH and Cys58 of GR.[21] However, in ajoene-GR, the S_γ atom of Cys58 is rotated by 90° when compared to GSH-GR which leads to positions of the ligand sulfur atoms differing by 2.58 Å and concomitantly distinct protein contacts. In the ajoene-modified enzyme, the ligand sulfur is in hydrogen-bonding distance to the OH group

of Ser30 (3.6-Å distance) and 5.2 Å from NE of His467′. In the GSH-GR structure, the S_γ of CysI forms a hydrogen bond with the NE of His467′ (distance 3.4 Å) but is 5.29 Å apart from the OH group of Ser30. The distance between the OH group of Ser and the sulfur atom of the ajoene fragment falls perfectly into the range of hydrogen bonds between OH groups and half-cystine residues in proteins.[28] A second direct contact with the protein may be formed via the sulfoxo group which is in hydrogen-bonding distance to the main chain nitrogen of Ala34. This nitrogen atom also participates in the fixation of glutathione in GSH-GR.[21] In modified trypanothione reductase, the ligand sulfur is suggested to be also in hydrogen-bonding distance to the OH group of Ser30 since this residue is conserved in the active site of both flavoenzymes. In contrast to the inhibition of GR, the reaction between ajoene and free cysteine leads to the formation of allyl−S−S−cysteine, and a mixed disulfide involving the 1,5-dithiaocta-2,7-diene 5-oxide fragment has not been observed.[25]

GR which is covalently modified by ajoene shows an increased oxidase activity. Blocking of Cys58 is expected to shift electron density toward the flavin ring promoting reduction of molecular oxygen. A comparable observation has been made for quinone reduction by GR and TR. The presence of $NADP^+$ which increases the electron density of the flavin ring in the EH_2 form enhances the proportion of one- over two-electron reduction of quinones.[29] The elevated oxidase activity of ajoene-modified GR, together with its inability to reduce glutathione disulfide, should have a strong impact on the redox status of the cell. Inhibitors of GR and TR with this profile have been termed "turncoat inhibitors" or "subversive substrates".[30−32] (For reviews, see refs 13 and 15.) They lower the GSH/GSSG and $T(SH)_2/TS_2$ ratio, respectively, waste NADPH, and produce reduced oxygen species. To our knowledge, ajoene is the first example of a turncoat inhibitor which inhibits the reductases covalently. The observation that ajoene-treated *T. cruzi* cells show a marked shift from unsaturated to saturated fatty acids in their phospholipid composition[5] is consistent with oxidative stress being involved in the antiparasitic effect of the compound.

In addition to being a covalent inhibitor of the enzymes, ajoene is a substrate of GR and TR leading to single- and two-electron-reduced products (Table 2). The bimolecular steady-state rate constants for the reduction of ajoene by GR and TR are comparable to those reported for the reduction of *p*-quinones by yeast GR and *T. congolense* TR.[29]

Reduction of (*Z*)-ajoene by GR yields deoxyajoene (compound **2**) and the symmetrical disulfide (compound **5**) as stable reaction products (Figure 4). The two compounds are also the main hydrophobic products in the in vitro reaction between ajoene and GSH and cysteine, respectively, at pH 8.0.[25] In the spontaneous reaction, formation of compound **5** is pH-independent, but large amounts of compound **2** are only found under alkaline conditions. In the GR-catalyzed reaction raising the pH from 6.9 to 7.4 does not change the reaction products formed. The pronounced reactivity of GR toward ajoene is probably due to the low p*K* values of the active site thiols being about 6.2 and 4.8 for Cys58 and Cys63, respectively.[12] The main products (compounds **2** and **5** in Figure 4) in the reduction of ajoene by GR show that both the disulfide and the sulfoxo group are reduced by the enzyme. The latter reaction occurs most probably via formation of a sulfoxide−thiol adduct which reacts with another thiol (Cys63) to form the thioether.



This mechanism has been shown for many model systems, and the reaction rate is highly dependent on the acidity of the thiol.[33] An alternative mechanism could involve formation of a sulfenate intermediate (R−SO^-) which reacts with the second protein thiol to form disulfide, thioether, and water.[33] In any case, the same reaction products, deoxyajoene and $NADP^+$, would be formed.

GR shows a distinct behavior toward (*E*)- and (*Z*)-ajoene isomers. (*Z*)-Ajoene is the favored substrate, whereas inhibition is slightly faster and more complete in the case of (*E*)-ajoene. These differences may reflect the preferred binding mode of the respective isomer in the active site. Since the ajoene fragment covalently linked to Cys58 (Figures 2 and 3) still possesses the sulfoxo group, one may speculate that reduction of the disulfide bridge as the first step favors inhibition of the enzyme. In contrast, if the sulfoxo group is reduced first, the preferred reactions may be those depicted in Figure 4. It should be mentioned that the different behavior of GR toward (*E*)- and (*Z*)-ajoene is only kinetic in nature, since both isomers are substrates as well as inhibitors of the enzyme. From a structural point of view the respective (*Z*)-ajoene-derived fragment could also be accommodated in the active site of GR.

In contrast to GR, TR does not discriminate between (*E*)- and (*Z*)-ajoene, and inhibition and reduction occur at lower rates. For many types of inhibitors of TR and GR, charge has been shown to be the major discriminating factor.[34] Since ajoene is an uncharged molecule, the wider active site of the parasite enzyme[35] may be the main reason for the similar reactivity with both isomers.

Conclusions

Human glutathione reductase and *T. cruzi* trypanothione reductase are attractive target molecules for the rational development of antitumor and antiparasitic drugs.[13,15,36] The structure of GR inhibited by ajoene shows for the first time the interaction of this garlic-derived natural compound with a target protein in atomic detail. Covalent modification of an active site cysteine residue of GR results in an enzyme species with

pronounced oxidase activity. In addition, ajoene acts as a substrate of the flavoenzymes leading to single-electron-reduced intermediates and the concomitant formation of superoxide anions. These results fit nicely with a recent report which showed that, in leukemic cells, ajoene induces apoptosis via generation of oxidative stress.[10]

The interactions of ajoene with GR and TR, key enzymes of the intracellular thiol metabolism, together with the direct effect of ajoene on the thiol homeostasis of the cell,[9] may at least in part be responsible for the cytostatic, antimalarial, and antitrypanosomal properties of this natural compound.

Experimental Procedures

Chemicals and Enzymes. (*E*)- and (*Z*)-Ajoene were prepared according to published procedures[37] and purified on a prepacked silica gel column (LiChroprep Si 60, 25 × 310 mm; Merck) with 20% 2-propanol in *n*-hexane; 10 mM stock solutions were made in ethanol using absorption coefficients at 240 nm for (*E*)- and (*Z*)-ajoene of 8.9 and 8.6 mM^{-1} cm^{-1}, respectively.[37] The solutions were stored at −20 °C. Diallyl trisulfide (CH_2═CH−CH_2−S−S−S−CH_2−CH═CH_2; Tiang Pharmaceutic Factory, Shanghai, PR-China) was kindly provided by Dr. Ronald Kaminsky, Swiss Tropical Institute, Basel, Switzerland. The concentration was determined photometrically at 210 nm (ϵ = 11.4 mM^{-1} cm^{-1}) or at 240 nm (ϵ = 2.3 mM^{-1} cm^{-1}).[37] Trypanothione disulfide was purchased from Bachem, Switzerland. All other reagents were of the highest available purity. Recombinant human glutathione reductase[38] and *T. cruzi* trypanothione reductase[35,39] were prepared as described. Bovine superoxide dismutase (SOD) was from Serva, and horse heart cytochrome *c* was from Calbiochem.

Glutathione Reductase Assay. All kinetics were carried out in GR assay buffer (20.5 mM KH_2PO_4, 26.5 mM K_2HPO_4, 200 mM KCl, 1 mM EDTA, pH 6.9) at 25 °C as described.[38] The standard assay mixture contained 100 μM NADPH and 5−10 mU GR. The reaction was started by adding 1 mM glutathione disulfide (GSSG), and the absorption decrease at 340 nm due to NADPH consumption was followed.

Trypanothione Reductase Assay. TR activity was measured in TR assay buffer (40 mM Hepes, 1 mM EDTA, pH 7.5) as described.[31] The standard assay mixture contained 100 μM NADPH and 5−10 mU *T. cruzi* TR. The reaction was started by adding 120 μM trypanothione disulfide (TS_2), and the absorption decrease at 340 nm was followed. V_{max} is calculated using a K_m value of 18 μM for TS_2.[31]

Cytochrome *c* Reduction. The ajoene- and DATS-mediated reduction of cytochrome *c* (Fe^{3+}) to cytochrome *c* (Fe^{2+}) was followed at 550 nm (ϵ = 19.1 mM^{-1} cm^{-1}). In a total volume of 100 μL, 1−2 U GR was incubated with 200 μM NADPH and 25 μM cytochrome *c* in assay buffer, pH 7.5, for 2 min at 25 °C. The reaction was started by adding 200 μM ajoene or DATS. Control assays contained 200 μM NADPH, 25 μM cytochrome *c*, and ajoene or DATS but no enzyme. The generation of superoxide anion radicals during the reaction was tested by adding 5 μg of superoxide dismutase.

Inhibition of GR and TR by Ajoene. GR (200 μL) (0.4−1.0 U) in assay buffer was incubated with 200 μM NADPH and varying concentrations (12.5, 50, 100, and 200 μM) of (*E*)- and (*Z*)-ajoene. A control sample contained all components except the inhibitor was replaced by ethanol. At different time intervals 2-μL aliquots of the reaction mixture were removed, and the remaining enzyme activity was determined in a standard GR assay.

TR (200 μL) (0.4−1.0 U) was incubated with 200 μM NADPH and 200 μM (*E*)- and (*Z*)-ajoene, respectively. Aliquots were removed and assayed as described for GR.

To prepare a large sample of (*E*)-ajoene-inhibited GR for crystallization, 70 mL of GR (5 U/mL) was incubated with 200 μM NADPH and (*E*)-ajoene. After 30 min another 200 μM NADPH and (*E*)-ajoene were added. After 90 min the reaction mixture was concentrated in a Centriprep-10 concentrator (Amicon) to a final volume of about 1 mL. The protein was precipitated by 90% ammonium sulfate, centrifuged, and dialyzed against GR assay buffer.

Reduction of (*Z*)-Ajoene by GR and Analysis of the Reaction Products by NMR. In a total volume of 50 mL, GR (3.3 U/mL) was incubated with 1 mM NADPH and (*Z*)-ajoene. After 20 min at 25 °C another 1 mM NADPH and 22 U GR were added. After 45 min the turbid reaction mixture was centrifuged at 2000 rpm for 60 min, and the precipitate was dissolved in acetonitrile. The reaction products were separated by HPLC applying 200-μL samples onto a C-18 Spherisorb column (MZ Analysentechnik Mainz) equilibrated in acetonitrile/water (v/v 60/40). Elution was achieved isocratically at a flow rate of 1.5 mL/min. Peaks were detected by absorption measurement at 210 nm; 1-mL fractions were collected. The HPLC-separated reaction products were diluted with water until the development of turbidity indicated precipitation of the water-insoluble compounds. The suspensions were applied onto Sep-pack cartridges (1 mL) and washed with deuterated water in order to remove water and acetonitrile. The compounds were then eluted with deuterated acetonitrile. The NMR spectra were kindly recorded by Dr. Horst Groβkurth on 360- and 500-MHz NMR instruments (Bruker) at the Max-Planck-Institut für Medizinische Forschung, Heidelberg.

Crystallization of (*E*)-Ajoene-GR and Data Collection. Solutions of human GR (7.5 and 10 mg/mL) which had been inhibited by (*E*)-ajoene to 70−90% were dialyzed against 5% ammonium sulfate in GR assay buffer. The protein was crystallized using the hanging drop procedure; 10-μL droplets were equilibrated with 1 mL of 17% ammonium sulfate in GR assay buffer. Orange crystals of 250 × 150 × 125 μm^3 grew within 120 days at 4 and 25 °C. Diffraction data were collected at 4 °C in the dark using a three-circle goniometer and Cu Kα radiation generated by a rotating anode (Molecular Structure Corp.) equipped with a graphite monochromator and recorded by a X1000 HiStar detector (Siemens). The data were reduced in space group $P2_1$ and $C222_1$ with the program XDS.[40,41] The data statistics are shown in Table 1.

Refinement. The crystal structure of human glutathione reductase inhibited by (*E*)-ajoene was determined by the molecular replacement method using the program package AMoRe.[42] The structure of the mixed disulfide between glutathione reductase and GSH (Protein Data Bank code 1GRE[21]) was used as a search model for both space groups ($P2_1$ and $C222_1$). Rotation and translation functions yielded clear maxima for a dimer and a monomer, respectively. The refinement (without σ cutoff) was performed with X-PLOR 3.851 and included bulk solvent correction, the standard protocol for conjugate-gradient minimization and slow cooling,[43] and refinement of individual temperature factors. To reveal a putative asymmetric modification of the two active sites of the homodimeric enzyme, the initial refinements were carried out in space group $P2_1$ (which contains a dimer in the asymmetric unit) and in space group $C222_1$ (containing a monomer in the asymmetric unit). Refinement in $P2_1$ was performed by imposing noncrystallographic symmetry restraints between the two monomers in the asymmetric unit. No restraints were applied for residues within an 8-Å sphere centered at Cys58, the expected binding site of ajoene. $(F_{obs} - F_{calc})$ exp $i\alpha_{calc}$, $(2F_{obs} - F_{calc})$ exp $i\alpha_{calc}$, and $(3F_{obs} - 2F_{calc})$ exp $i\alpha_{calc}$ electron density maps were inspected with the interactive graphics program O.[44] As no significant differences were found between the two monomers in space group $P2_1$ (especially around Cys58), the final steps of the refinement were carried out in $C222_1$. However, it should be noted that the true space group is $P2_1$, since the two subunits of human GR are interconnected by a disulfide bridge between Cys90 and Cys90′.[45]

After the protein structure was rebuilt, water molecules with temperature factors < 45 $Å^2$ and finally the inhibitor fragment were incorporated. Since reduction of ajoene by GR yields several products, three different fragments bound to Cys58 of the protein were analyzed. Finally for the inhibitor

fragment 2, which best explained the electron density, several orientations were tested (see Results). The stereochemistry of the model is good with deviations of 0.009 Å in bond lengths and 0.925° in angles (Table 1).

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Supporting Information Available: Spectrum of (*E*)-ajoene-inhibited human glutathione reductase. This material is available free of charge via the Internet at http://pubs.acs.org.

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