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Enzymatic Activity of the *Arabidopsis* Sulfurtransferase Resides in the C-Terminal Domain But Is Boosted by the N-Terminal Domain and the Linker Peptide in the Full-Length Enzyme

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Sulfurtransferases/rhodaneses are a group of enzymes widely distributed in plants, animals, and bacteria that catalyze the transfer of sulfur from a donor molecule to a thiophilic acceptor substrate. Sulfurtransferases (STs) consist of two globular domains of nearly identical size and conformation connected by a short linker sequence. In plant STs this linker sequence is exceptionally longer than in sequences from other species. The *Arabidopsis* ST1 protein (AJ131404) contains five cysteine residues: one residue is universally conserved in all STs and considered to be catalytically essential; a second one, closely located in the primary sequence, is conserved only in sequences from eukaryotic species. Of the remaining three cysteine residues two are conserved in the so far known plant STs and one is unique to the *Arabidopsis* ST1. The aim of our study was to investigate the role of the two-domain structure, of the unique plant linker sequence and of each cysteine residue. The N- and C-terminal domains of the *Arabidopsis* ST1, the full-length protein with a shortened linker sequence and several point-mutated proteins were overexpressed in *E. coli*, purified and used for enzyme activity measurements. The C-terminal domain itself displayed ST activity which could be increased by adding the separately prepared N-terminal domain. The activity of an ST1 derivative with a shortened linker sequence was reduced by more than 60% of the wild-type activity, probably because of a drastically reduced protein stability. The replacement of each cysteine residue resulted in mutant forms which differed significantly in their stability, in the specific ST activities, and in their kinetic parameters which were determined for 3-mercaptopyruvate as well as thiosulfate as sulfur substrates: mutation of the putative active site cysteine (C332) essentially abolished activity; for C339 a crucial role at least for the turnover of thiosulfate could be identified.

Key words: *Arabidopsis thaliana* / 3-Mercaptopyruvate / Mutagenesis / Sulfurtransferase / Thiosulfate.

Introduction

Sulfurtransferase enzymes catalyze the transfer of a sulfur atom from suitable sulfur donors to nucleophilic sulfur acceptors. The most studied and best characterized sulfurtransferase (ST) is bovine rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.1.1) which catalyzes, *in vitro*, the transfer of a sulfane sulfur atom from thiosulfate to cyanide, leading to the formation of sulfite and rhodanide. In this reaction rhodanese cycles between the sulfur-free (*E*) and sulfur-substituted (*E*S) form. Solution studies have identified three catalytic requirements: an active site sulfhydryl group, two cationic residues, and a hydrophobic environment for substrate binding (Finazzi Agro *et al.*, 1972; Miller-Martini *et al.*, 1994a,b).

Bovine rhodanese is a nuclear-encoded monomeric protein of approximately 32 kDa which is localized in the mitochondria. The tertiary structure of bovine rhodanese is composed of two domains which, in spite of negligible sequence homology, are characterized by very similar three-dimensional folds; the two domains are connected by a loop at the surface of the molecule (Russell *et al.*, 1978; Ploegman *et al.*, 1978). The active site is located in the C-terminal domain, near the interface between the two domains. Site-directed mutagenesis studies of bovine rhodanese have shown that out of four conserved and reduced cysteines, C247 is the only cysteine required for the activity of the enzyme (Miller-Martini *et al.*, 1994a). A persulfide link between this essential sulfhydryl group and the transferred sulfur is formed in the *E*S intermediate (Ploegman *et al.*, 1979; Gliubich *et al.*, 1996).

In many prokaryotes and eukaryotes 3-mercaptopyruvate (3-MP) sulfurtransferase activity (MST, EC 2.8.1.2) was discovered. The MST protein is located in the cytosol and in the mitochondria of the eukaryotic cell (Wood and Fiedler, 1953; Meister *et al.*, 1954; Westley, 1973; Nagahara *et al.*, 1999). The enzyme was purified to homogeneity (Nagahara *et al.*, 1995) and cloned from rat liver (Nagahara and Nishino, 1996). Both ST proteins, MST and rhodanese, isolated from the same organism (*Rattus*) accepted 3-MP and thiosulfate, but the ratios of their respective enzyme activities differed. However, the purified as well as the recombinant rat MST revealed K_m values

for 3-MP in the low millimolar range. Therefore, it is still an open question whether 3-MP is the naturally occurring substrate for this rat enzyme.

ST activity in cyanogenic and non-cyanogenic plants was detected many years ago (Chew, 1973), but only recently two *Arabidopsis* cDNA sequences encoding ST proteins were isolated and characterized (Hatzfeld and Saito, 2000; Nakamura *et al.*, 2000; Papenbrock and Schmidt, 2000a,b). On the amino acid level both protein sequences show very high homology to each other (81%) and resemble the MST from mammals with respect to decisive amino acid residues. However, the number and positions, and therefore maybe also the function, of most of the cysteine residues differ in plant ST sequences from known ST sequences in other organisms. Both *Arabidopsis* STs display similar substrate specificities. The specific activities are 5–10 times higher with 3-MP than with thiosulfate whereas the K_m is about 5 times lower for thiosulfate than for 3-MP (Papenbrock and Schmidt, 2000b). So far the search for a true thiosulfate sulfurtransferase (TST) in *Arabidopsis* that shows homology to the mammalian TSTs by cDNA library screening and database comparisons was not successful.

Apart from two-domain STs for which the length and composition of the linker sequences connecting the domains were found to differ, a number of sequences from several organisms were identified which show homologies to either the N-terminal or the C-terminal domain only. For example, the 12 kDa GlpE protein from *E. coli* resembles the active site domain of mammalian rhodaneses and shows ST activity probably in its dimeric form (Ray *et al.*, 2000). In *Arabidopsis* a number of proteins with homologies to either the N-terminal or the C-terminal domain were identified (Papenbrock and Schmidt, 2000b). One of these smaller proteins might act as a specific TST in plants. These observations raised the question whether each domain of the *Arabidopsis* ST might form an active ST enzyme by itself.

Although STs are present in many types of organisms from all three domains of life, their physiological functions are still in question. Proposed roles include cyanide detoxification (Vennesland *et al.*, 1982), sulfur metabolism (Donadio *et al.*, 1990), and mobilization of sulfur for iron-sulfur cluster biosynthesis or repair (Bonomi *et al.*, 1977; Pagani *et al.*, 1984). In plants also the mobilization of sulfur for transport processes in older leaves was suggested (Papenbrock and Schmidt, 2000b). What are the differences between plant ST proteins and mammalian and prokaryotic STs? Answering this question might also help to clarify the function of STs in the plant organism which might be divergent from the function in vertebrates and bacteria.

The purpose of this study was to work out the special characteristics of one particular plant ST (ST1) in comparison to STs from other organisms. Therefore, the ST1 protein with a shortened linker sequence and both domains were separately expressed, purified and analyzed for their enzymatic activities. To elucidate the importance of each single cysteine residue in ST1, the point mutated versions of ST1 were studied with respect to their stabilities, their substrate binding properties, and activities. Two substrates, thiosulfate as well as 3-MP, were used to investigate the influence of the different bond cleavage types (cleavage of S-S or C-S bond).

Results

Primary Structure and Activity Features of the ST1 Protein from *Arabidopsis*

In the databases the identical ST proteins from *Arabidopsis* were named either TSTs (Hatzfeld and Saito, 2000) or MSTs (Nakamura *et al.*, 2000; Papenbrock and Schmidt, 2000a,b). Careful analysis of the older data and results presented here lead us to rename the proteins into the more general term ST because the natural substrate might be neither 3-MP nor thiosulfate.

The ST1 chosen for this study is a nuclear encoded protein which is transferred into mitochondria. The deduced amino acid sequence of this protein (accession no. AJ131404) has five amino acid replacements and one deletion compared with formerly reported *Arabidopsis* sequences (accession nos. AB032864, AJ011045 and the completed genome sequence) of the cultivar Columbia. The clone we work with was isolated from an *Arabidopsis* cDNA library of which the cultivar is not known (Papenbrock and Schmidt, 2000a). However, the cultivar is obviously divergent from Columbia because the DNA sequence was checked by sequencing in both strands more than once. The mature ST1 protein consists of 321 amino acids and has a predicted molecular mass of 35.51 kDa.

It contains 5 cysteine residues, one in the N-terminal part and four in the C-terminal part (Figure 1). The protein sequences of STs from different groups of organisms were aligned (Papenbrock and Schmidt, 2000a) and the positions of cysteine residues in the sequences were compared. In ST1 the active site cysteine is residue C332 when counting begins with the first amino acid of the full-length protein including the transit peptide. The cysteine residue C152 is restricted to ST1 and ST2 from *Arabidopsis*, C294 can only be found in the four so far known plant sequences, C304 is unique in the ST1 protein, whereas C339 is highly conserved in all eukaryotic ST proteins.

Enzymatic Activities of the N- and C-Terminal Domains of the *Arabidopsis* ST1

The STs from several organisms consist of two domains consisting of different amino acid sequences whereas the three-dimensional structures are very similar. Thus, we were interested in potential enzyme activities of the N- and C-terminal domains of the *Arabidopsis* ST1. Both domains were expressed in *E. coli* with an N-terminal His_6-tag attached and purified to homogeneity by nickel-affinity chromatography. Of each protein 0.5 µg was loaded

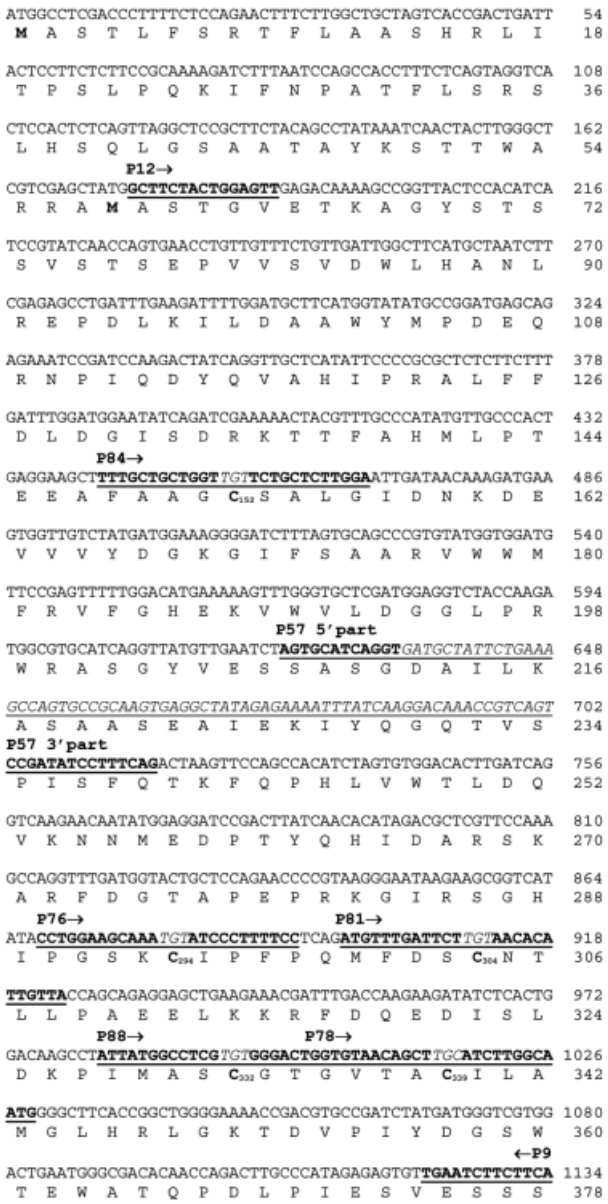

Fig. 1 cDNA and Protein Sequences of Sulfurtransferase 1 (ST1).
The full-length cDNA and the translated amino acid sequence of the ST1 from *Arabidopsis thaliana* (accession no. AJ131404) are shown. The sense primers and the sequence of antisense primer 9 are given; only the primer parts which are completely homologous to the sequence are printed in bold letters. The numbers (P#) correspond to the respective primers listed in Table 1. The codons that were changed in the mutagenesis experiments are given in italic letters. The cysteine residues which were exchanged for the amino acids indicated in Table 1 are printed in bold. The part of the linker sequence between the N-terminal and the C-terminal part which was deleted is shown in italic letters. The methionine residues indicating the start of the full-length protein and of the mature protein are in bold face letters.

on an SDS-PAGE and visualized with Coomassie Brilliant Blue (Figure 2, lanes 8 and 9), which revealed a high degree of purity (at least 95%). The sizes of the purified recombinant proteins were in agreement with the predicted

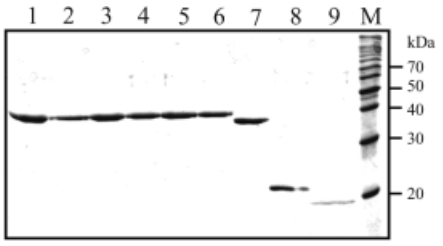

Fig. 2 SDS-PAGE of the Different Recombinant ST Proteins Investigated in This Study.
The mutant forms of ST1 containing an N-terminal His_6-tag were expressed in *E. coli* and purified by nickel-affinity chromatography. One µg of each protein or 0.5 µg in the case of STN-term and STC-term were loaded on an SDS-PAGE and stained with Coomassie Brilliant Blue. The SDS-PAGE was loaded from the left to the right in the following order: lane 1, ST1; lane 2, STC152A; lane 3, STC294N; lane 4, STC304E; lane 5, STC332S; lane 6, STC339V; lane 7, STwlink; lane 8, STN-term; lane 9, STC-term. The names for the proteins are listed in Table 1. The size of the molecular masses of standard proteins in kDa is indicated on the right side of the gel.

molecular masses including the His_6-tag referring to standard proteins. The purified proteins were dialyzed overnight at 4 °C and were directly used for enzyme activity determinations (Figure 3). At least two independent isolates were examined and the activity measurements were done in duplicate.

The N-terminal part (STN-term) alone did not show MST activity at all (Figure 3A), whereas the C-terminal part (STC-term) which contains the active site cysteine was able to catalyze the sulfur transfer from 3-MP to cyanide. The enzymatic activity was about 0.5% when compared to that of ST1, but well measurable whereas transfer of sulfur from thiosulfate was not detectable.

In a reconstitution experiment increasing amounts of the STN-term domain were added to the STC-term peptide (Figure 3A). The ratios given at the bottom of the graph are mg protein STN-term per mg protein STC-term. The more of the STN-term peptide was added in proportion to the STC-term domain, the higher the enzyme activity calculated on the basis of the protein amount of the STC-term domain. At a protein ratio of 5 to 1 or 10 to 1 (STN-term to STC-term) the specific activities were about 10% of the wild-type activities (compared with values shown in Figure 4). It was also investigated whether the enzyme activity of wild-type ST1 could be increased by adding the N- or C-terminal domains in varying amounts (Figure 3B). Neither a surplus of the N-terminal domain nor of the C-terminal domain could increase the overall activity of the wild-type protein significantly.

Investigation of the Role of the Prolonged Linker Sequence

In plant ST sequences the connecting linker sequence between both domains is 21 amino acids longer than in various ST sequences from prokaryotic and other eukaryotic species. To obtain more information about the

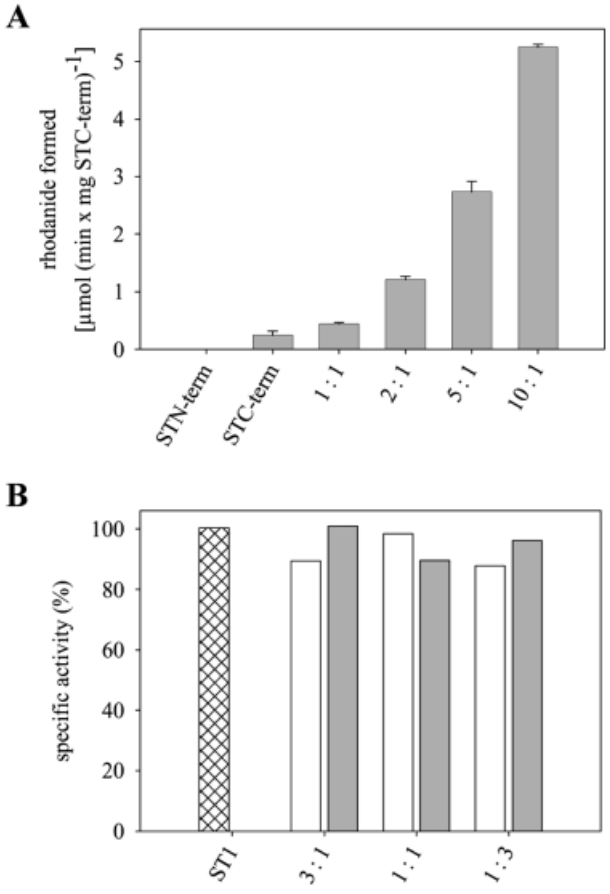

Fig. 3 Investigation of the Enzymatic Properties of the N- and the C-Terminal Domain.
(A) The N-terminal and the C-terminal parts of the ST1 protein were expressed separately in *E. coli* and purified. Each protein fragment was used for enzyme activity measurements using 3-MP as a substrate (STN-term, STC-term). Additionally different ratios of the two protein fragments were combined to determine the specific enzyme activity calculated on the basis of the protein amount of the STC-term domain. The numbers at the bottom of the columns give the ratios of the STN-term protein to the STC-term protein in mg protein to mg protein. (B) The specific enzyme activities of the wild-type protein using 3-MP as substrate (right bar) and in combination with varying amounts of the STN-term domain and the STC-term domain, respectively, are given (white bars, wild-type protein in combination with the STN-term domain; gray bars, wild-type protein in combination with the STC-term domain).

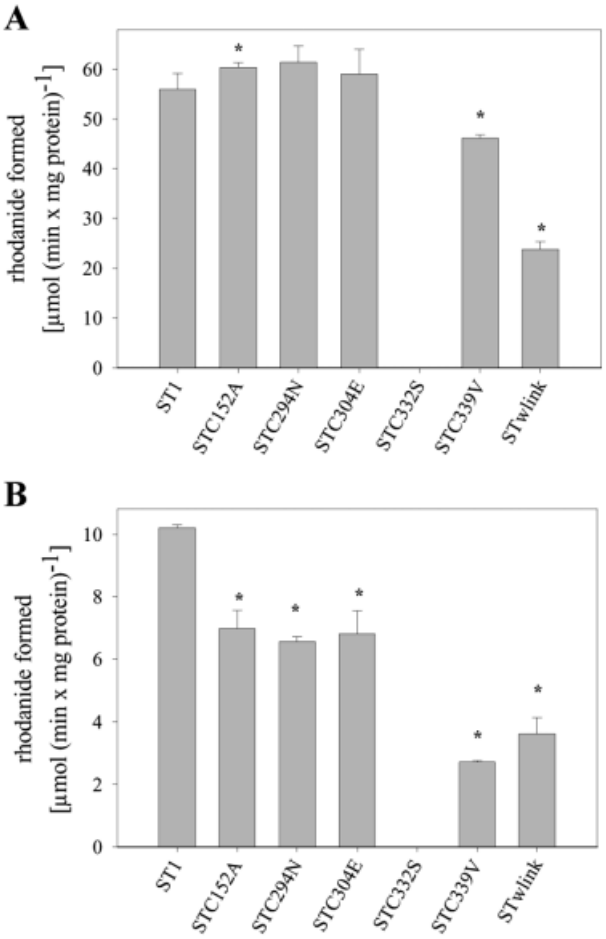

Fig. 4 Enzyme Activity Measurements of the Mutant Forms of ST1.
The recombinant STs were purified as described in Materials and Methods, dialyzed overnight and then directly used for enzyme activity measurements. One µg of recombinant protein was used routinely in each assay. At least two independent isolates were examined and the activity measurements were done in duplicate or triplicate. The specific activities that are significantly different at $p < 0.05$ from the wild-type ST1 activity are marked with an asterisk. (A) 3-MP was used as sulfur donor. (B) Thiosulfate was used as sulfur donor. TS, thiosulfate.

function of this linker sequence a peptide of 23 amino acids was deleted from ST1 (STwlink). This deletion halved the specific enzyme activity with 3-MP in comparison to the specific activity of wild-type ST1 (Figure 4A). When thiosulfate was used the STwlink protein had only about one third of the wild-type activity (Figure 4B). To determine the overall stability of the STwlink protein the effects of pre-incubation at elevated temperatures (39 and 41 °C) in comparison to regular assay temperature (37 °C) and of added urea (2.5 and 4 M) were investigated using 3-MP as substrate (Figure 5). The STwlink protein was very sensitive to these conditions: it lost up to 50% of its activity by a pre-incubation at 41 °C in comparison to pre-incubation at 37 °C, whereas ST1 lost only about 15% of its activity. After incubation with 4 M urea only 10% residual activity was found with STwlink but still 50% of activity with ST1. Obviously, the linker sequence plays not only an important role in providing a hydrophobic environment for substrate binding and catalysis as was shown previously (Finazzi Agro *et al.*, 1972; Miller-Martini *et al.*, 1994a,b) but is also highly responsible for the overall stability of the ST protein.

Enzyme Activities and Stabilities of Several Mutant Forms

Table 1 and Figure 1 give an overview about the different clones containing amino acid replacements. In the clones produced for this study the cysteine residues at the respective positions were replaced by the amino acids

Table 1 Summary of the Clones Produced and Primers Used.

Clone name	Changed bases	Primer sequence
STC152A	453 – 455	P84 (P3) 5'-TTT GCT GCT GGT GCA TCT GCT CTT GGA-3'
STC294N	882 – 884	P76 (P3) 5'-CCT GGA AGC AAA AAT ATC CCT TTT CC-3'
STC304E	912 – 914	P81 (P3) 5'-ATG TTT GAT TCT GAG AAC ACA TTG TTA-3'
STC332S	996 – 998	P88 (P3) 5'-ATT ATG GCC TCG TCA GGG ACT GGT GTA-3'
STC339V	1014 – 1016	P78 (P3) 5'-GGT GTA ACA GCT GTC ATC TTG GCA ATG-3'
STwlink	634 – 702	P57 (P3) 5'-AGT GCA TCA GGT CCG ATA TCC TTT CAG-3'

The numbers of the cysteine residue exchanged and the respective amino acids introduced are indicated in the first column. The second column gives the positions of the bases which were changed in the cDNA sequence. The third column shows the primer pairs used for mutagenesis. Only the sequence of the sense primer (P3) is listed, the respective primers 4 are complementary to the primer 3 sequences. In clone STwlink a part of the linker sequence between the N-terminus and the C-terminal domain (23 amino acids) was deleted.

Table 2 Determination of the K_m Values for the Substrates 3-Mercaptopyruvate and Thiosulfate, the Catalytical Constants k_{cat} and the Quotients k_{cat}/K_m Using the Different Recombinant Sulfurtransferase Proteins.

Protein	Mol. mass (kDa)	3-Mercaptopyruvate			Thiosulfate		
		K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m
ST1	35.51	3.40	33.1	9.7	0.25	6.0	24.0
STC152A	35.47	2.89	35.7	12.4	2.22	4.1	1.8
STC294N	35.52	2.47	36.3	14.7	0.22	3.9	17.70
STC304E	35.52	3.64	34.9	9.6	0.64	4.0	6.3
STC332S	35.49	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
STC339V	35.50	2.32	27.3	11.8	1.33	1.6	1.2
STwlink	33.13	8.66	13.2	1.5	1.43	2.0	1.4

Enzyme assays were done as described in Materials and Methods. Each determination was done four times. The molecular masses ('Mol. Mass') were calculated for the recombinant proteins excluding the His_6-tag. 'n.d.': not detectable.

conserved in the animal MSTs. The wild-type ST1 and the five mutant forms were expressed, purified, and checked for impurities by SDS-PAGE analysis (Figure 2). The protein names and the predicted molecular masses of the mutant forms are listed in Table 2. The purified proteins were dialyzed overnight at 4 °C and were directly used for enzyme activity determinations (Figure 4). When the cysteine residues number 152, 294, or 304 were replaced the specific activities with 3-MP were the same or even slightly higher than the specific activity of the wild-type ST [50 µmol (min × mg protein)$^{-1}$] (Figure 4A). When the C332 which is supposed to be part of the active site was replaced the enzyme activity was undetectable whereby the detection limit was determined to be about 10 nmol (min × mg protein)$^{-1}$ in the rhodanide assay. Replacement of C339 by valine led to a small but significant decrease in the specific activity.

Thiosulfate was also studied as sulfur donor substrate. In general, the specific activity with thiosulfate was reduced to about 20% in comparison to the MST activity [10 µmol (min × mg protein)$^{-1}$] (Figure 4B). Proteins STC152A, STC294N, and STC304E showed a significantly reduced activity (down to about 70%) in comparison to the wild-type ST1. The specific activity of protein STC339V was drastically reduced to 25% residual activity which contrasts to the slight reduction when 3-MP was used as substrate. Again the mutant form STC332S was inactive. A comparison of the ratios of the specific activities with 3-MP or thiosulfate revealed that the most discriminating effect with respect to these substrates could be observed with protein STC339V; the relative specific activity of STC339V was 3 times higher when 3-MP was used as the sulfur substrate.

Again the effects of elevated temperatures and increased urea concentrations on the enzyme activities using 3-MP as substrate were determined (Figure 5). Mutants STC304E and STC339V were more susceptible to higher temperature than the other mutant forms and the wild-type ST1. The activities of ST1 and STC152A were reduced significantly only after pre-incubation at 41 °C (Figure 5A). The reduction in enzyme activity of the mutant forms at 2.5 M urea varied between 20% (STC294N) and 50% (STC304E) and at 4 M between 50% (ST1) and 80% (STC339V) (Figure 5B). The conformation of most

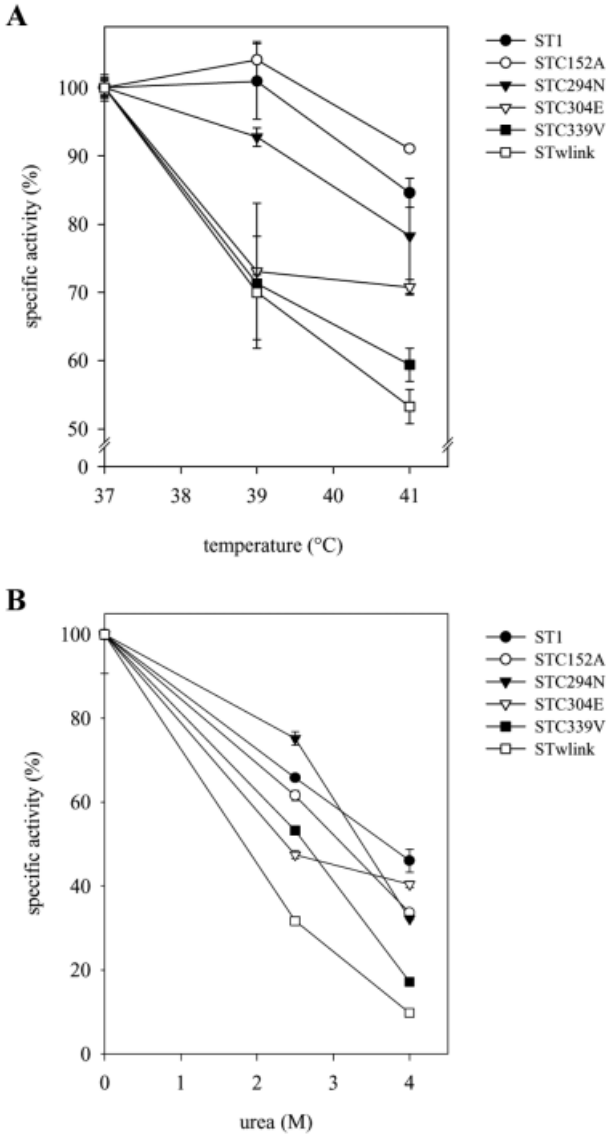

Fig. 5 Stability of the Mutant Proteins in Comparison to the ST1 Wild-Type Protein.
(A) Susceptibility to thermal denaturation. All protein solutions were incubated at the temperatures indicated for 10 min. After the pre-incubation the enzyme activity was assayed at 37 °C using 3-MP. The activity of each protein pre-incubated at 37 °C was set to 100%, the other values refer to this control activity. (B) Susceptibility to urea denaturation. The different protein solutions were incubated with 2.5 or 4 M urea for 10 min at room temperature. The enzyme activity was subsequently assayed with 3-MP as substrate. The activity of each protein without urea treatment was set to 100%. Both experiments were repeated three times independently in duplicates.

mutant forms seems to be less stable than wild-type ST1 at increased urea concentrations, indicating an unfolding under these conditions.

Kinetic Parameters of Wild-Type and Mutant Proteins

To compare the substrate affinities and specificities of wild-type ST1, the mutant forms and STwlink, the kinetic parameters were determined (Table 2). The calculation of the catalytical constant (k_{cat}) reveals the velocity of product formation. It gives the theoretical maximum of the molar activity. High k_{cat} values indicate good substrates. With 3-MP as substrate the k_{cat} values were more or less the same for the wild-type and the mutants STC152A, STC294N, and STC339E; with thiosulfate the wild-type protein displayed an about 1.5fold higher k_{cat} value than these mutants.

The catalytic efficiency (k_{cat}/K_m) includes both the enzyme activity and substrate affinity and therefore takes into account the impact of the mutation on activity and substrate binding. This quotient is a valuable tool for the comparison of different substrates for the same protein with respect to their physiological properties, and therefore of the substrate quality. It is decreased dramatically for the STwlink protein with both substrates. Interestingly, the k_{cat}/K_m values of wild-type ST1 and the various point mutant proteins are in the same range for 3-MP but differ significantly for thiosulfate as substrate, where the catalytic capabilities of the STC339V and also of the STC152A mutant are most significantly impaired.

To investigate the role of C339 in more detail the K_m values for cyanide using wild-type ST1 and STC339V protein were determined. The K_m for cyanide of STC339V was 1.5 times higher than for ST1 in presence of either 3-MP or thiosulfate. The activation of the ST1 protein with 2-mercaptoethanol with respect to the specific activity measured with 3-MP (Papenbrock and Schmidt, 2000a) was 50% more efficient than in the presence of thiosulfate, whereas we did not observe any difference in the activation of STC339V by 2-mercaptoethanol between both substrates. In a fractionation experiment using gel-exclusion chromatography protein solutions of ST1 and STC339V were analyzed. For both protein preparations two fractions were eluted, one containing the monomer and the other the dimeric form. Both protein fractions revealed the same specific ST activity. Therefore, the C339 seems not to be involved in intramolecular disulfide bridge formation.

Discussion

We were interested in a detailed investigation of the ST1 from *Arabidopsis* because of some remarkable differences in the primary structure in comparison to STs from other organisms. All experiments described were undertaken with proteins containing a His_6-tag at the N-terminus. It was shown that the *Azotobacter* ST with a His-tag and the native *Azotobacter* ST purified by conventional gel-exclusion chromatography were indistinguishable in their kinetic parameters (Pagani *et al.*, 2000). Therefore, we assume that the additional histidines at the N-terminus do not disturb our observations. The C-terminus might be more susceptible to any perturbations (Kramer *et al.*, 2001). Almost all STs investigated so far accepted both substrates, 3-MP and thiosulfate, however, the re-

spective K_m values differed. Only the *Azotobacter* ST showed unique properties among the characterized STs because it accepted almost exclusively thiosulfate with a 1×10^3 fold higher specific activity than with 3-MP (Colnaghi *et al.*, 1996). Probably the unique stretch of amino acids around the active site is responsible for this substrate specificity (Bordo *et al.*, 2000; Pagani *et al.*, 2000).

For bovine rhodanese it was shown by solution and X-ray studies that C247 is essential for covalent sulfur binding. When the sulfur-free rhodanese was inactivated by phenylglyoxal in the presence of cyanide disulfide bonds were formed between C247 and C254 (70%) and C247 and C263 (30%), two of the 4 cysteine residues present (Weng *et al.*, 1978; Miller-Martini *et al.*, 1994a,b). These results raised the question whether cysteine residues other than the active site cysteine contribute to ST function. It was demonstrated in mutagenesis studies with bovine rhodanese that C247 is the only sulfhydryl group that is necessary and sufficient to catalyze the transfer of sulfur *in vitro*, the non-catalytic sulfhydryl groups influence the stability, folding, and oxidative susceptibility of bovine rhodanese (Luo *et al.*, 1994; Miller-Martini *et al.*, 1994a; Kramer *et al.*, 2001).

In summary, the comparison of the kinetic parameters revealed that the specific activity of ST1 was much higher when 3-MP was used as sulfur donor in comparison to thiosulfate. However, the K_m value for thiosulfate was much lower and finally the k_{cat}/K_m value was higher than with 3-MP. Both substrates could be metabolized naturally, but the physiological levels of 3-MP are rather low in comparison to the K_m determined (Papenbrock and Schmidt, 2000b); the kinetic data may therefore indicate that better substrates still need to be found. The k_{cat} values of the *Arabidopsis* ST1 were also lower than those determined for STs from other organisms (Westley, 1973; Alexander and Volini, 1987; Nagahara *et al.*, 1995, 1999; Ray *et al.*, 2000) which might again indicate a divergent substrate specificity. From the point mutant studies C332 was proven to be an essential residue with both substrates used. The effects of the other mutations on the kinetic parameters were more pronounced with the thiosulfate substrate; substitution of C339 severely reduced k_{cat}/K_m as well as k_{cat}, substitution of C152 strongly reduced k_{cat}/K_m. C339 also proved to be critical for the stability of the enzyme and its mutation increased the K_m for cyanide which might point to a general importance of this residue for ST1 activity.

The reaction mechanisms of STs are still a matter of debate: for both types, TST and MST, different reaction mechanisms were suggested. MST is supposed to transfer the sulfur in a single displacement from 3-MP to cyanide as acceptor whereas TST was suggested to follow a double displacement mechanism (Nagahara *et al.*, 1995, 1999). The *Arabidopsis* protein obviously accepts both substrates and requires C332 to process both, however, the mechanism has not been clarified by our experiments.

A closer look at the amino acids which correspond to the replaced cysteine residues in *Arabidopsis* ST1 in the three-dimensional model of bovine rhodanese (Gliubich *et al.*, 1996) shows that C294 and C304 are probably located at the surface of the protein molecule, C152 could be close to the surface. C332 and C339 are buried in the molecule in close neighbourhood to each other, however, probably without direct interaction. This speculative comparison underlines the putative special role of C339 in the reaction mechanism.

In the bovine rhodanese the interdomain linker between the globular domains consists of 16 extremely hydrophobic amino acids. The linker in the STs from *Arabidopsis* and *Datisca* has a similar hydrophobicity but is extended by 21 amino acids to a complete linker length of 37 amino acids. We were interested in the role of the rather long insertion which is unique to plant STs. In former experiments two amino acids were inserted into the bovine rhodanese through site-directed mutagenesis to introduce a new V8 cleavage site. The specific activity was only slightly reduced to about 90% of wild-type activity whereas the stability and conformational transitions were dramatically effected (Luo *et al.*, 1995). Fujita and coworkers (2000) investigated the role of the interdomain linker sequence in *E. coli* RNA polymerase with respect to enzyme activity by detailed mutagenesis studies: it was observed that rather the overall three-dimensional structure of the linker than the position of certain amino acids facilitated correct positioning of both domains.

The removal of the plant specific linker-part led to a reduction in the enzyme activity to about 30% for both substrates. Also the stability of the protein with the shortened linker was drastically reduced. The prolongation of the linker during evolution of the plant protein was obviously essential to provide full enzyme activity according to our *in vitro* results. Because of the longer linker also the function in the plant organisms could be diverse from other organisms. One could speculate that the true substrate in plants might be larger than 3-MP or thiosulfate and therefore the nearby active site needs to be protected and surrounded by a larger hydrophobic environment. If the ST1 should be involved in the activation of proteins by the direct transfer of a sulfane sulfur the extended hydrophobic hollow space might be necessary for close contact between the active site and the protein of interest.

In several organisms homologs to both the N- and C-terminal domains of ST were isolated (Papenbrock and Schmidt, 2000b; Ray *et al.*, 2000; Wrobel and Czubak, 2000). The 12 kDa *E. coli* protein GlpE shows similarity to the C-terminal part of ST and revealed high ST activity by its own. One might ask the question whether the N-terminal domain of those ST proteins consisting of two domains is needed at all for enzyme activity, and if yes, what is the function of this domain. We could demonstrate that the C-terminal domain of the *Arabidopsis* protein alone showed very low MST, but no detectable TST activity. In the databases at least two proteins were identified from *Arabidopsis* which reveal similarity to the C-terminal domain of ST and are good candidates for true TSTs al-

though they are much smaller than the *Arabidopsis* STs isolated so far.

Addition of increasing amounts of the separately prepared and per se inactive N-terminal domain to the isolated C-terminal domain which contains the active site led to a linear increase in the specific activity of the C-terminal domain. The probability that a domain pair forms a protein complex might increase over the concentration range studied. One has to bear in mind that none of the two domains possesses the plant specific linker sequence which could explain the low affinity and the lower activity as compared to full length ST1. A surplus of neither the N-terminal nor the C-terminal domain improved or inhibited the overall performance of the wild-type ST1 which means that the structure and stoichiometry of the wild-type protein are 'optimal' under the conditions investigated. A transgenic approach by expressing the mutated or truncated enzymes in an *Arabidopsis* ST knockout mutant could provide evidence for the relevance of our results *in vivo*.

Materials and Methods

DNA Cloning and Mutation Techniques

To produce point-mutated ST cDNA clones the *in vitro* mutagenesis PCR method described by Innis *et al.* (1990) was followed. The cDNA coding for the mature ST protein was amplified using primer 12 (= primer 1) extended by a *Kpn*I restriction site (5'-CAG GTA CCG CTT CTA CTG GAG TT-3') and primer 9 (= primer 2) extended by a *Pst*I restriction site (5'-CGC TGC AGT CAT GAA GAA GAT TCA-3') and cloned into the pQE-30 expression vector (Qiagen, Hilden, Germany) as described before (Papenbrock and Schmidt, 2000a). This clone was used as a template for the amplification of the 3'-fragment by primer 2 and the respective primer 3 and for the amplification of the 5'-fragment by primer 1 and the respective primer 4 (complementary sequence of primer 3) (Table 1). After removal of the primers the full-length mutated PCR fragment was amplified using the heteroduplex molecules as template by primers 1 and 2 and finally cloned into the pQE-30 vector, resulting in proteins carrying an N-terminal His_6-tag. The successful replacement of the respective base pairs was proven by sequencing. The expression clone without the coding region of 69 bp for the prolonged plant-specific linker (STwlink) was produced by the same PCR method with the primers given in Table 1.

In general, for each PCR reaction 0.2 mM dNTPs (Roth, Karlsruhe, Germany), 0.4 µM of each primer (MWG, Ebersberg, Germany), 1 mM $MgCl_2$ (Roth) (final concentration, respectively), 0.75 µl RedTaq DNA-Polymerase (Sigma, Taufkirchen, Germany) and about 1 µg template-DNA in a final volume of 50 µl was used. Before starting the first PCR cycle, the DNA was denatured for 180 s at 94 °C followed by 28 PCR cycles conducted for 60 s at 94 °C, 60 s at the respective annealing temperature (between 49 °C and 54 °C) and 60 s at 72 °C. The process was finished with an elongation phase of 420 s at 72 °C. In the amplification reaction of the heteroduplex molecule the amount of template DNA was increased to 3 µg, in the first 5 cycles the annealing temperature was lowered to 44 °C and the prolongation time was extended to 90 s.

The N-terminal and the C-terminal domains of the mature ST protein were ligated into the pQE-30 vector excluding the prolonged plant linker sequence. The N-terminal part (STN-term) consisting of 462 bp was amplified using primer 12 and primer 74 (5'-CCT GCA GAC CTG ATG CAC TAGA-3'), the C-terminal part (STC-term) consisting of 432 bp with primer 9 and primer 75 (5'-CGG TAC CCC GAT ATC CTT TCA GA-3').

Expression and Purification of the ST Proteins in *E. coli*

The respective expression vectors were transformed in the *E. coli* strain XL1-blue. Most of the recombinant proteins could be expressed following this standard protocol: after growth of the respective *E. coli* cultures at 37 °C to an OD_{600} of 0.6 in Luria Bertani medium (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 10 g l^{-1} NaCl) (Roth) containing ampicillin (100 µg ml^{-1}) (AppliChem, Darmstadt, Germany) induction was carried out for 3 h with 1 mM final concentration of isopropyl-β-D-galactoside (AppliChem). To avoid the formation of inclusion bodies the induction of the STN-term protein was carried out at 30 °C for 3 h. Cell lysis was obtained by adding lysozyme (final concentration 1 mg ml^{-1}) (Roth) and vigorous homogenizing using a glass homogenizer and a pistil. The recombinant proteins were purified under non-denaturing conditions by affinity chromatography with the nickel-affinity resin according to manufacturer's instructions (Qiagen). Most of the recombinant protein eluted at 250 mM imidazole, 300 mM NaCl, 50 mM NaH_2PO_4 (Roth). The eluted fractions were dialyzed overnight with excessive amounts of 20 mM Tris/HCl, pH 7.5 at 4 °C. Between 1 and 40 mg of the respective recombinant purified protein was obtained from 1 l of *E. coli* cultures.

SDS Polyacrylamide Gel Electrophoresis

Denaturing gel electrophoresis of purified protein solutions was done according to Laemmli (1970). Protein samples were heated at 95 °C in loading mix (90 mM Tris/HCl, pH 8.0, 20% glycerol, 2% SDS, 0.02% bromophenol blue, 100 mM dithiothreitol) for 15 min and directly loaded onto the SDS gel; proteins were visualized by Coomassie Blue staining.

Enzyme Activity Measurement

Affinity purified protein was tested for enzyme activity as described (Papenbrock and Schmidt, 2000a). In general, 1 µg purified protein was used in the enzyme assays. The determination of the K_m values was done in 1 ml assays under the following conditions: 0.1 M Tris/HCl pH 9.0, 10 mM KCN and 5 mM 2-mercaptoethanol (Fluka, Taufkirchen, Germany) using 12 different substrate concentrations in the range of 100 µM to 27.5 mM of 3-MP (ICN, Eschwege, Germany) or thiosulfate (Fluka), respectively. To investigate the stability of the mutant forms the influence of elevated temperature (37, 39, and 41 °C) and denaturation by 2.5 and 4 M urea was tested in pre-incubation experiments. For this purpose, purified protein (1 mg ml^{-1}) was pre-incubated for 10 min at the temperatures indicated or with the respective concentration of urea. Subsequently the specific activity was determined as described above.

Miscellaneous

Protein concentrations were determined according to Bradford (1976) using bovine serum albumin (Roth) as a standard. The DNA and amino acid sequence analyses and prediction of the molecular masses were performed with the programs Protean and MapDraw in DNASTAR (Madison, WI, USA). Statistical analysis was performed using the Student method (SigmaPlot for Windows version 7.0). The K_m values were calculated from the non-linear Michaelis-Menten plot using an enzyme kinetics program (SigmaPlot 7.0).

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