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Properties of thiols required for sulfur dioxygenase activity at acidic pH

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In mesoacidophilic sulfur-oxidizing bacteria, such as *Acidithiobacillus* and *Acidiphilium* spp., elemental sulfur is oxidized by a thiol-dependent dioxygenase to sulfite. The thiols form with elemental sulfur highly reactive sulfanes, which are the actual substrate of the dioxygenase. Thus far, the identity of the required thiols is unknown. Consequently, the classical *in vitro* enzyme assay which is generally based on the addition of glutathione (GSH) is unsuitable for characterizing enzymatic sulfur oxidation in these bacteria. In this study, we present data on *in vitro* pH dependence of sulfur dioxygenase from *Acidiphilium acidophilum* DSM 700, indicating a limitation at the lower pH range caused by, besides other experimental factors, the quite high pI of the added GSH (which is around 3). It is speculated that thiols with pIs of about 2 could extend dioxygenase activity below the observed pH limit of about 3.5–4. The demonstration of significant sulfur dioxygenase activity at pH values around 2 would support the hypothesis of a periplasmic localization of this enzyme in Gram-negative mesoacidophilic sulfur oxidizers which generally have an acidic periplasm and a neutral cytoplasm. Furthermore, designing an enzymatic assay more adapted to the intracellular conditions would help to identify and characterize the components (thiols and proteins) which are *in vivo* responsible for the transport, activation, and oxidation of elemental sulfur.

Keywords: *Acidiphilium*; organic polysulfane; elemental sulfur; glutathione; sulfur dioxygenase

1. Introduction

Acidophilic sulfur-oxidizing bacteria such as *Acidithiobacillus ferrooxidans* and the closely related *At. thiooxidans*, as well as *Acidiphilium acidophilum* and other mesophilic species, play an important role in bioleaching of metal sulfides. These bacteria are regularly found at mining sites and biohydrometallurgical operations (for the extraction of metals such as copper, cobalt, uranium, gold, etc.) when moderate temperatures prevail (*1*). The heavy metal sulfides are oxidized via intermediate sulfur species, such as elemental sulfur and polythionates ($S_nO_6^{2-}$, $n > 2$), to sulfuric acid (*2*). Due to the high stability of elemental sulfur under mesoacidophilic conditions (about 20–40 °C and pH below 3), elucidation of the mechanism of bacterial sulfur oxidation would be of use in understanding the efficiency of the leaching process (*3*). Consequently, an understanding of the microbial and biochemical fundamentals is desired for controlling this important reaction in bioleaching operations (*4*).

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Since the pioneering work of Isamu Suzuki and coworkers, it is known that a thiol-dependent dioxygenase is catalyzing the oxidation of elemental sulfur in mesophilic leaching bacteria (*5–7*). Unfortunately, even after nearly 50 years of research, the identity of the responsible enzyme components remains unknown. In addition, the mechanism of the dioxygenase-catalyzed reaction is still not resolved. Recently, we have proposed a model for elemental sulfur oxidation in *Acidithiobacillus* and *Acidiphilium* spp. (*8*). In this scheme, extracellular sulfur reacts with thiol groups (P-SH) of outer membrane proteins (Equation 1), is transported into the periplasmic space in the form of highly reactive sulfane sulfur (S_n with $n \geq 1$ in P-SS_nH, *e.g.*, with $n = 1$ the persulfide P-SSH) and is then oxidized by sulfur dioxygenase to sulfite (Equation 2).

$$S_8 + \text{P-SH} \longrightarrow (\text{P-}SS_8H) \longrightarrow \text{P-}SS_nH \tag{1}$$

$$\text{P-}SS_nH + O_2 + H_2O \longrightarrow \text{P-}SS_{n-1}H + SO_3^{2-} + 2H^+. \tag{2}$$

Our conclusions are based on investigations following glutathione (GSH)-dependent elemental sulfur oxidation in cell-free extracts by quantifying all relevant inorganic and organic sulfur compounds, including reaction products of GSH with elemental sulfur, bisorganylpolysulfanes, GS_nG with $n = 2$–5 (*8*). In this test, GSH plays the role of the thiol-bearing proteins (P-SH) and reacts with elemental sulfur to form glutathione persulfide (GSSH) or higher homologues, which are the actual substrate of sulfur dioxygenase (*8*). The GS_nG species result from reactions of monorganylpolysulfanes (GSS_nH) with GSH or other GSS_nH species. In the case of two persulfide molecules (GSSH), for example, the trisulfane GS_3G is formed (Equation 3).

$$2\text{GSSH} \longrightarrow GS_3G + H_2S. \tag{3}$$

According to this model, the thiol groups responsible for elemental sulfur activation and transport, as well as the sulfur dioxygenase, have to be active at low pH values as extracellular and periplasmic pH is normally around 2 in acidophilic bacteria. In contrast, thus far reported pH optima of sulfur dioxygenase are around 7 and the lowest value where activity was observed is around pH 5 (*9*). However, this pH dependency was not determined under physiological conditions but only obtained with crude extracts or partially purified enzymes using an *in vitro* assay system. Generally, the thiol GSH is added in the activity test as sulfur dioxygenase of mesophilic leaching bacteria is dependent of externally added thiols. Although GSH is a biogenic thiol, it is unlikely that this low molecular weight compound is able to carry out the complex task of sulfur activation and transport across cell membranes and in the periplasmic space under acidic conditions. We speculate that integral membrane and periplasmic proteins with thiols as functional groups adapted to low pH values are active *in vivo*. Consequently, previous results based on assays where GSH or similar low molecular weight thiols have been applied do not reflect the physiological conditions of acidophilic sulfur-oxidizing bacteria.

Here, we present further results on the pH dependence of the sulfur dioxygenase obtained from *Ap. acidophilum* DSM 700 in the widely used GSH-dependent enzyme assay. Implications based on properties of the *in vitro* added thiol compound are discussed and ideas on the nature of the *in vivo* responsible thiol group containing proteins are presented.

2. Materials and methods

2.1. *Chemicals*

Sulfur compounds were purchased from Merck (sublimed elemental sulfur 'Schwefelblüte', potassium or sodium salts of sulfide, thiosulfate, sulfite, and sulfate) or Sigma (reduced and

oxidized glutathione, GSH and GSSG, respectively). Other chemicals used in the study were purchased from Merck. All chemicals were at the highest purity available and were used as received without further purification.

2.2. *Bacterial strain and cultivation*

In this study, *Ap. acidophilum* DSM 700 obtained from the DSMZ (Braunschweig, Germany) was used. It was grown on a medium according to Mackintosh (*10*) with the following modifications: 1 mM $(NH_4)_2SO_4$ was substituted by 2 mM NH_4Cl, and the initial pH was adjusted to 3.0 with hydrochloric acid instead of sulfuric acid. As substrate, 5 g of elemental sulfur powder per liter was added. The strain was cultivated aerobically at 28°C.

2.3. *Cell harvesting and disruption*

All steps were performed at ≤4 °C. Cultures in the late logarithmic growth phase were concentrated by filtration and remaining elemental sulfur was removed by centrifugation at 120*g* for 5 min. Cell pellets were obtained by centrifugation at 10,000 g for 10 min and washed twice in a salt solution (107 mg NH_4Cl, 25.4 mg $MgCl_2 \times 6\ H_2O$, and 147 mg $CaCl_2 \times 2\ H_2O$ per liter, adjusted to pH 3.0 with hydrochloric acid). The final cell pellet was suspended in a phosphate buffer (50 mM, pH 6.5) at densities of about 25 g cell protein per liter. The disruption procedure was performed under an oxygen-free atmosphere of $N_2/CO_2/H_2$ (88:10:2, v/v/v) in an anaerobic workstation. An equivalent volume of glass beads (150–212 μm, Sigma) was added to this suspension and then stirred for 1 h. Afterwards, the liquid phase was decanted, filled up with the same volume of phosphate buffer (50 mM, pH 6.5), and centrifuged (20 min, 25,000*g*, twice) for removing cells and cell residues. The supernatant, in the following referred to as crude or cell-free extract, usually contained 2 g protein per liter and was stored under an oxygen-free atmosphere ($N_2/CO_2/H_2$) at −25°C.

2.4. *Enzyme assay*

Cell-free extracts were tested for sulfur dioxygenase activity according to Suzuki (*6, 9*) with minor modifications. All assays were performed aerobically at 30°C with stirring at 300 rpm. The reaction mixtures contained 10 mM phosphate buffer adjusted to final pH values of 3–9 as indicated, crude extract (0.01 or 0.02 g bacterial protein per liter), dispersed elemental sulfur (4 mM) and GSH (varying from 0.2 to 5 mM as indicated). For stabilizing the dispersed elemental sulfur particles, 0.2 g per liter of bovine serum albumin (BSA) was added when assays were adjusted to pH values ≤5.5. The initial pH was maintained by titration with 50 mM KOH or 50 mM hydrochloric acid, resulting in a deviation of ≤0.2 pH units from the target pH value during experiments. In order to determine non-enzymatic reactions, assays with heat-inactivated crude extracts (after incubation at 90°C for 30 min) were used. Samples were analyzed for sulfur species (see below). With the exception of elemental sulfur quantification, all samples were filtered (nylon filter, 0.2 μm) prior to sulfur analyses in order to remove suspended sulfur. Sulfur-oxidizing activity is given as the amount of sulfur atoms that have been oxidized from the zero valence state to at least the oxidation state of sulfite. The finely dispersed elemental sulfur was prepared by mixing deionized water with an equal volume of acetonic sulfur solution (saturated solution containing about 20 mM elemental sulfur). For acetone removal, the mixture was then dialyzed against deionized water. The dialysis product contained about 6 mM elemental sulfur, forming droplets of 2–10 μm in diameter.

2.5. *Analyses of sulfur compounds*

For analyzing sulfur species, two chromatographic systems were used. Thiosulfate, GSH, and GSH derivatives (GS_nG species with $n = 2$–5 and $GSSO_3^-$, *i.e.* glutathione S-sulfonate) were analyzed by ion pair chromatography. A HPLC system from Kontron/BIO-TEK Instruments was applied, with a guard column cartridge PLRP-S 5 × 3 mm and a separation column PLRP-S 100 Å, 8 μm, 150 × 4.6 mm (Latek Labortechnik, Germany), and a diode array detector. Chromatograms were recorded at 205, 215, 265, and 300 nm concomitantly with spectra from 190 to 320 nm. An eluent consisting of an aqueous solution of 18% acetonitrile, 2 mM tetrabutylammonium chloride, and 2 mM acetate buffer at pH 4.0 was pumped isocratically at 1 mL/min. Chromatograms recorded at 215 nm were used for quantification. Due to the lack of appropriate standards, higher homologues of GSSG could not be determined at molar concentrations. Therefore, only peak areas were recorded. Identification of higher bisorganylpolysulfanes (GS_nG) was achieved on account of their spectra (Figure 1) and their specific retention characteristics in an isocratic chromatographic system, as independent of the HPLC system used for separation of homologous bisorganylpolysulfanes a linear relation is found between number of sulfur atoms and logarithm of capacity factor (*11, 12*). A calibration for the GSH S-sulfonate was achieved by monitoring the stoichiometric conversion of sulfite plus GSSG to GSH S-sulfonate plus GSH (Equation 4) by analogy with conversion of other disulfides such as cystine (*13, 14*) under anaerobic conditions to prevent autoxidation of sulfite. In a typical calibration experiment, GSSG was incubated with sulfite in a phosphate buffer (10 mM, pH 7.2) for about 3 h at 25°C (Figure 2). For quantification, peak areas recorded at 215 nm were used. Elemental sulfur was analyzed by reversed-phase chromatography followed by UV-detection at 254 nm as previously described (*15*). One volume of sample was diluted with five volumes of ethanol and injected directly. In case of low concentrations of elemental sulfur, samples were extracted with *n*-octane or isooctane prior to dilution with ethanol. Sulfite, thiosulfate and sulfate were quantified by ion exchange chromatography and conductivity detection as previously described (*15*). A Dionex DX 500 system with a guard column AG9-SC/4 mm and a separation column AS9-SC/4 mm was applied. Sulfite was stabilized by adjusting the pH of samples to pH 11 with a 100 mM KOH solution and adding 0.05% (v/v)

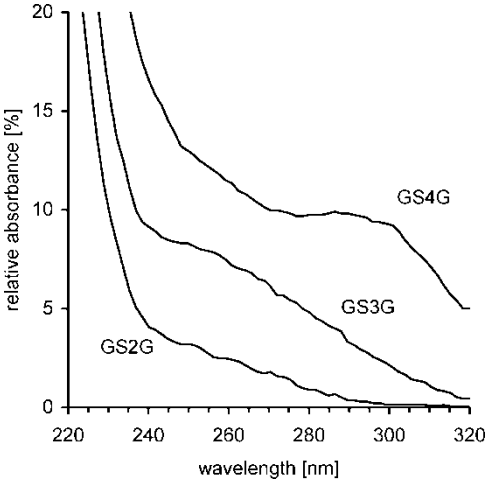

Figure 1. UV spectra of bisorganylpolysulfanes of GSH (GS_nG, $n = 2$–4), recorded after chromatographic separation with the HPLC system described in Section 2.5. The relative absorbance was calculated by setting the value recorded at 205 nm to 100%.

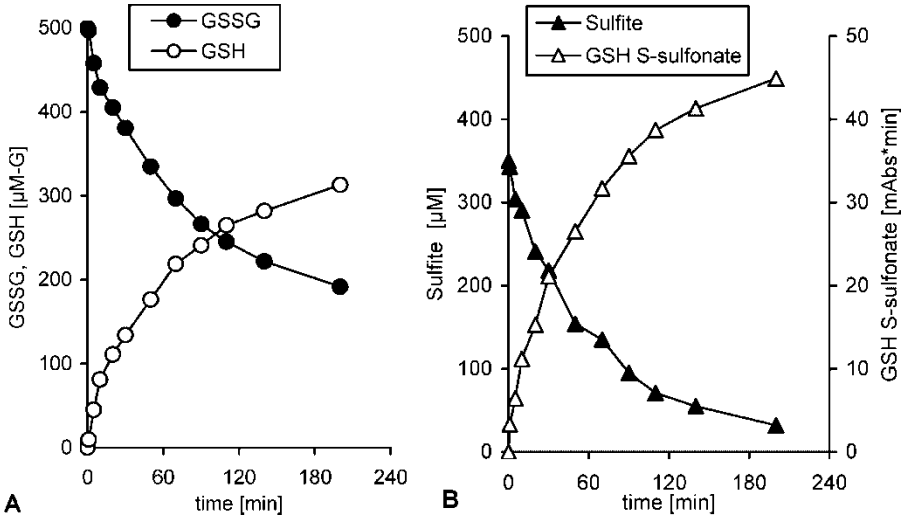

Figure 2. Non-enzymatic formation of glutathione S-sulfonate ($GSSO_3^-$) according to Equation 4. Here, 0.25 mM GSSG were incubated with 0.35 mM sulfite under anaerobic conditions at pH 7.2 and 25°C. Standard deviation of replicates was within 5% (not shown). (A) GSSG and GSH concentrations are given in μM-G, considering the number of glutathione moieties in these compounds. (B) Sulfite and $GSSO_3^-$ concentrations. Amounts of $GSSO_3^-$ are given as the peak areas recorded at 215 nm.

of a 37% methanal solution (*16, 17*).

$$GSSG + SO_3^{2-} + H^+ \longrightarrow GSH + GSSO_3^-. \quad (4)$$

Dissolved sulfide was determined photometrically by the methylene blue method as previously described (*8*). Samples were fixed with zinc acetate and stored frozen or analyzed immediately.

2.6. *Other analytical procedures*

Protein concentration was determined by the method of Bradford (*18*), modified by Spector (*19*) using BSA as protein standard. The pH was measured potentiometrically.

2.7. *Statistical analyses*

Experimental data such as concentrations of sulfur compounds or enzymatic activities are given as mean values of at least five independent experiments with error bars representing the standard deviation. Linear regressions were performed for calibration and determination of enzymatic activity with the least squares method using the standard deviation of the individual values as weight. The significance of linear correlation was tested by t-test analyses.

3. Results

3.1. *pH dependence of sulfur dioxygenase activity*

The pH dependence of sulfur dioxygenase was determined with cell-free extracts from sulfur-grown cells of *Ap. acidophilum* DSM 700. In all cases, the main oxidation product was not sulfite but thiosulfate (Figure 3) as already found in a previous study (*8*). The latter sulfur compound

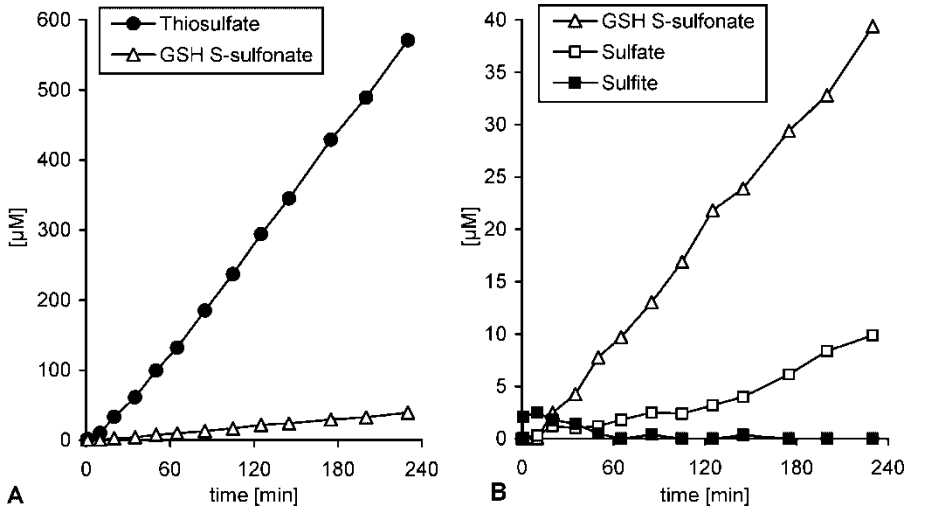

Figure 3. Speciation of oxidation products in the sulfur dioxygenase assay using a crude extract from *Ap. acidophilum* DSM 700 at pH 6.5 and an initial GSH concentration of 0.5 mM. The total amount of bacterial protein was 0.01 g/L. Standard deviation of replicates was within 5% (not shown). (A) Concentrations of thiosulfate and GSH S-sulfonate are given. (B) Concentrations of GSH S-sulfonate and sulfite as well as the increase in sulfate are given.

formed from sulfite in a non-enzymatical reaction with excess elemental sulfur according to Equation 5. Besides thiosulfate, minor oxidation products were GSH S-sulfonate, sulfate, and sulfite (Figure 3B).

$$1/8\,S_8 + SO_3^{2-} \longrightarrow S_2O_3^{2-}. \tag{5}$$

In a first approach, the assays were performed applying an initial GSH concentration of 0.5 mM. In this way, the pH optimum for enzymatic activity was obtained at around 7.5 (Figure 4).

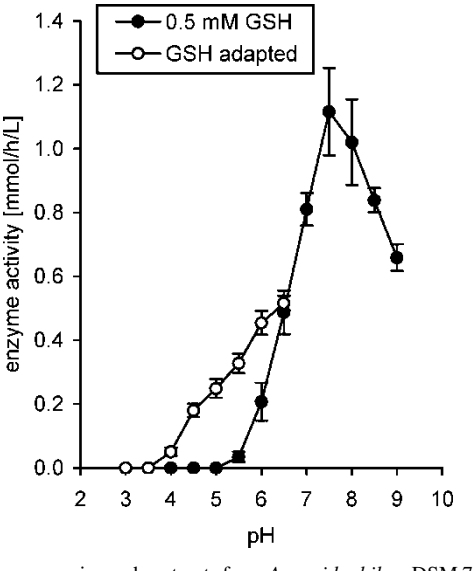

Figure 4. Activity of sulfur dioxygenase in crude extracts from *Ap. acidophilum* DSM 700 at various pH values. Closed circles represent the activity measured in the assay applying an initial GSH concentration of 0.5 mM. Open circles give the activity obtained when adapting the GSH concentration to decreasing pH values (1 mM at pH 5 to <6; 2 mM at pH 4 to <5; 5 mM at pH <4). In each assay, the total amount of bacterial protein was 0.02 g/L.

When increasing the pH to 9 and applying 0.5 mM GSH, still significant enzymatic sulfur oxidation occurred as it was only reduced to 50% of the maximum. The lowest pH where activity was detectable was around 5.5–6. In contrast, when applying higher initial GSH concentration than 0.5 mM, elemental sulfur was enzymatically oxidized at lower pH values. It turned out that while decreasing the pH the amount of GSH had to be steadily increased for obtaining enzymatic activity (Figure 4). In the range of pH 6.5–9, the addition of more than 0.5 mM GSH did not change oxidation activity. In addition, at a pH of 6.5–7 the initial GSH concentration could even be reduced to 0.2 mM without loss in activity (data not shown). On the other hand, between pH 5 and 5.5 and with 1 mM GSH, significant sulfur oxidation was observed while it was nearly absent when only applying 0.5 mM. Finally, by raising the GSH concentration up to 5 mM, the lower pH limit for enzymatic activity could be extended to 4. This clearly indicates that the amount of GSH is a limiting factor in this test system.

3.2. *pH dependence of GS_nG formation*

As glutathione persulfide and its higher homologues (GSS_nH) are the actual substrate for sulfur dioxygenase (*8*), it would be interesting to investigate the pH dependence of their synthesis in the assay system. Unfortunately, these organic sulfur species are instable and difficult to analyze. However, the formation of GS_nG species with $n > 2$ indicates the presence of GSS_nH species, as the former are reaction products of the latter (*e.g.* according to Equation 3). Hence, we tried to follow the formation of the higher homologues of GSSG. Due to the lack of appropriate standards, comparisons could only be made based on peak areas (recorded at 215 nm). In the absence of sulfur dioxygenase activity, formation of GS_nG species was maximal as GSS_nH species were only converted according to Equation 3 or analogous reactions to the bisorganylpolysulfanes of GSH and to H_2S. Then, the latter compound was regularly detectable in the assays at concentrations up to 100 μM (data not shown). Whereas high-dioxygenase activities resulted in smaller GS_nG and

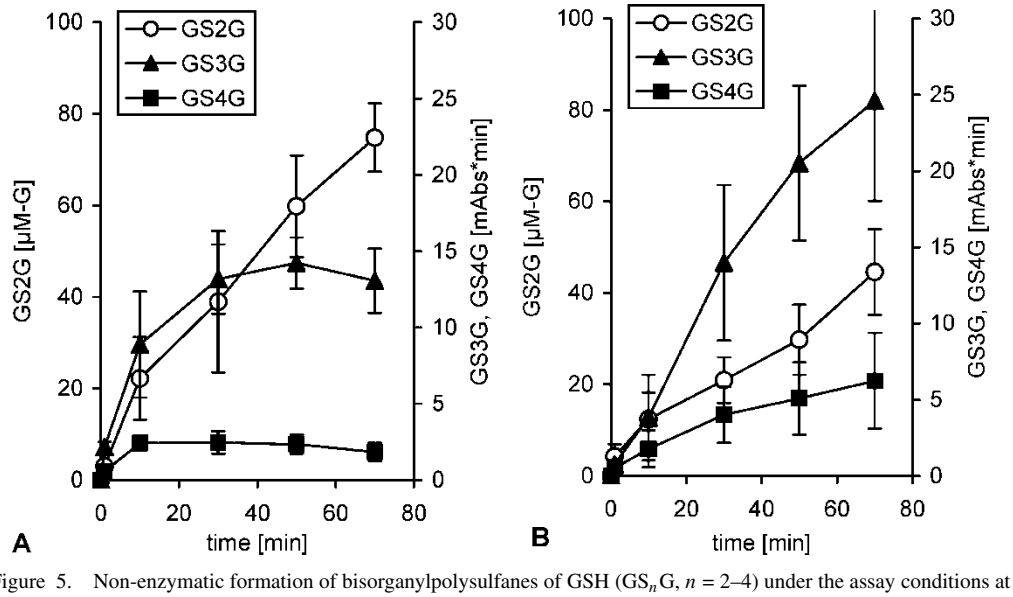

Figure 5. Non-enzymatic formation of bisorganylpolysulfanes of GSH (GS_nG, n = 2–4) under the assay conditions at two different initial GSH concentrations and pH values. (A) Initial GSH concentration was 0.2 mM at pH 7. (B) Initial GSH concentration was 5 mM at pH 3. Amounts of GS_3G and GS_4G are given as the peak areas recorded at 215 nm. The concentration of GSSG is given in μM-G, considering the number of glutathione moieties in this compound.

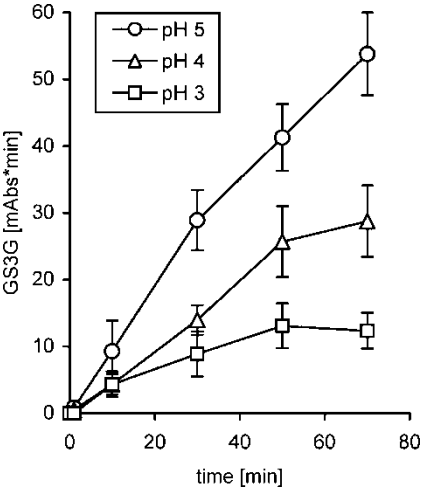

Figure 6. Non-enzymatic formation of GS_3G under the assay conditions at an initial GSH concentrations of 2 mM and varying pH values. Amounts of GS_3G are given as the peak areas recorded at 215 nm.

H_2S values due to the consumption of GSS_nH species in the oxygenation process by analogy with Equation 2 (*8*). Without enzymatic activity, however, GSH was rapidly oxidized to GSSG at pH 7 (Figure 5A). Within 70 min nearly 40% of the initially applied 0.2 mM GSH was converted to the disulfide. Besides, GS_3G and GS_4G were regularly detectable, showing a plateau after about 30 min of incubation (Figure 5A). In contrast, these GS_nG species could hardly be observed when 0.2 mM GSH was incubated at pH 3 (data not shown). However, they were detectable at this low pH when applying 5 mM GSH instead of 0.2 mM (Figure 5B). Under acidic conditions, although applying higher amounts of GSH, less GSSG but more GS_3G and GS_4G were produced than at pH 7. A comparison of GS_3G formation at various pH values and applying 2 mM GSH to the test system is shown in Figure 6. Generally, formation of higher bisorganylpolysulfanes decreased when lowering the pH.

4. Discussion

Although sulfur dioxygenase of acidophilic bacteria is postulated to be localized in the periplasm (*8*), having a pH of 2–3, enzymatic activities below pH 5 have not yet been observed in the GSH-dependent *in vitro* assay (*9*). By modifying the original test system in this study, the activity range of sulfur dioxygenase was extended to pH 4.

The observed demand for higher GSH concentrations at pH values below 6 for enzymatic activity can be partially explained with a reduced nucleophilicity of the thiol group under acidic conditions. However, the observed absence of enzymatic activity at pH 3.5 and below (Figure 4) is not due to GSH limitations as the formation of GS_nG species was still observed at pH 3 (Figure 5B). Probably, GSS_nH molecules, formed by analogy with Equation 1, are not available for enzymatic oxidation (Equation 2) due to competing abiotic reaction resulting in GS_nG formation (*e.g.* according to Equation 3).

GSH is a tripeptide with four pH-dependent functional groups, the thiol, the amino, and two carboxyl moieties (Figure 7). The pKa values of these groups reported first by Pirie and Pinhey in 1929 were 2.12, 3.53, 8.66, and 9.62 (*20*), resulting in a pI of 2.85. Hence, at neutral pH the thiol and amino groups are protonated while the two carboxyl moieties are negatively charged, giving a net charge of −1. In addition, at pH values above pI, GSH-derived compounds, meaning GSS_nH

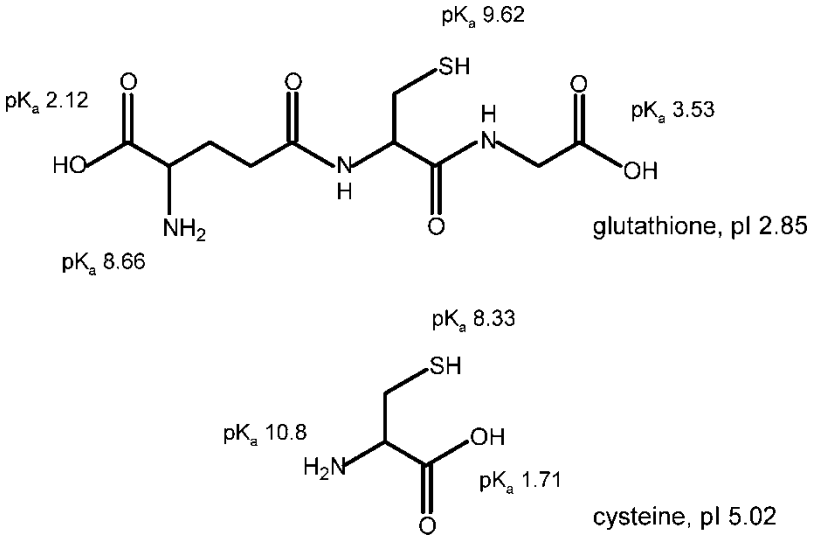

Figure 7. Structures and pKa values of glutathione and cysteine (*20*).

and GS$_n$G, are likewise negatively charged. This property may delay abiotic reactions of GSS$_n$H molecules at higher pH values due to repulsion forces between negatively charged areas near the thiol/sulfane groups, thus making sulfane sulfur atoms in GSS$_n$H available to sulfur dioxygenase. Moreover, it has been found that with the GSH alternative cysteine, which is not charged at the discussed pH range (Figure 7), only insignificant dioxygenase activities were measured (*6, 21*) which supports this hypothesis.

Further studies on the lower pH limit of sulfur dioxygenase are required as our findings indicate possible activities at pH 3 or lower. Thus alternative thiol compounds should be designed and tested in the enzyme assay. In particular, it is worthwhile to apply peptides that are negatively charged at low pH, for example, those having pI values around 2. In addition, research should be intensified for identifying the thiol compounds which are *in vivo* responsible for elemental sulfur activation and transport to sulfur dioxygenase. The search should focus on proteins of the outer membrane and periplasm with several cysteine residues, *e.g.*, proteins with similarity to mercury transport and reduction components. The latter have conserved motives with thiol pairs binding the heavy metal (*22, 23*). In mesoacidophilic sulfur-oxidizing bacteria, however, only a limited number of thiol-bearing periplasmic and outer membrane proteins have been characterized thus far. Possible candidates for activation and transport of elemental sulfur are the sulfide-binding protein isolated from *At. ferrooxidans* AP 19-3 (*24*) and several outer membrane proteins which have been associated with sulfur oxidation in strains of *At. ferrooxidans* (*25–27*). Moreover, other proteins originally found in neutrophilic bacteria might also be relevant for sulfur oxidation of leaching bacteria. One of these proteins is SoxY which has been described to bind sulfur compounds such as sulfide and thiosulfate by the thiol group of a conserved cysteine residue (*28*). SoxY is part of a periplasmic sulfur-oxidizing enzyme complex (Sox system) which has been well studied in the neutrophilic *Paracoccus pantotrophus* GB17 (*28*). Interestingly, in the genomes of *At. thiooxidans* ATCC 19377 and *At. caldus* ATCC 51756 a suite of genes have been identified with similarity to the Sox system (*29*). Future studies will show whether this system is active in these bacteria and in other acidophilic sulfur oxidizers.

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