Sulfide : quinone oxidoreductase (SQR) from the lugworm Arenicola marina shows cyanide- and thioredoxin-dependent activity

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The sulfide tolerance of marine invertebrates, such as the lugworm Arenicola marina, has been studied for many years. The animals live in marine sediments in which sulfide concentrations can reach up to 2 mM [1–3]. Sulfide is a potent toxin for humans and most animals, because it inhibits mitochondrial cytochrome c oxidase at micromolar concentrations, A. marina can use electrons from sulfide for mitochondrial ATP production. In bacteria, electron transfer from sulfide to quinone is catalyzed by the membrane-bound flavoprotein sulfide : quinone oxidoreductase (SQR). A cDNA from A. marina was isolated and expressed in Saccharomyces cerevisiae, which lacks endogenous SQR. The heterologous enzyme was active in mitochondrial membranes. After affinity purification, Arenicola SQR isolated from yeast mitochondria reduced decyl-ubiquinone (K_m = 6.4 μM) after the addition of sulfide (K_m = 23 μM) only in the presence of cyanide (K_m = 2.6 mM). The end product of the reaction was thiocyanate. When cyanide was substituted by Escherichia coli thioredoxin and sulfite, SQR exhibited one-tenth of the cyanide-dependent activity. Six amino acids known to be essential for bacterial SQR were exchanged by site-directed mutagenesis. None of the mutant enzymes was active after expression in yeast, implicating these amino acids in the catalytic mechanism of the eukaryotic enzyme.

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Abbreviations
Ni-NTA, nickel nitrilotriacetic acid; SQR, sulfide : quinone oxidoreductase.
[12], the corresponding DNA sequences or purified protein are lacking. A functional mitochondrial SQR that promotes electron transfer from sulfide to quinone was cloned and characterized from the fission yeast *Schizosaccharomyces pombe*, but the enzyme had very low affinities for sulfide and quinone, with $K_m$ values of 2 mM for both substrates. However, the *S. pombe* SQR showed marked sequence similarity to the bacterial SQR purified and characterized at the biochemical level from *Rhodobacter capsulatus* [13].

Sulfide:quinone oxidoreductase homologs have subsequently been reported in the genomes of many prokaryotes and eukaryotes, including fungi, insects and mammals [14]. Three distinct groups of sequence diversity (groups I, II and III) have been identified. Five SQR fingerprints have been identified for SQR bacterial group I. Three of these fingerprint domains, including two cysteines and the FAD-binding domain III, are conserved amongst all SQR sequences [14]. Sulfide:quinone oxidoreductase was probably an essential and ubiquitous enzyme during the phase of eukaryotic evolution 1–2 billion years ago, because the Earth’s ocean waters were anoxic and sulfidic during that time [15–17]. Even today, SQR is an important enzyme for many animals, because sulfide is produced endogenously in several tissues of mammals [18–20] and marine invertebrates [21]; in humans, the overproduction of sulfide can lead to disease [22]. However, from the standpoint of environmental ecology, modern sulfide-tolerant animals, such as *Arenicola*, require an enzyme for efficient sulfide oxidation. In this article, we report the isolation of an *sqr* gene from the sulfide-adapted, sand-dwelling marine worm *A. marina*, its heterologous expression in *Saccharomyces cerevisiae*, its kinetic parameters, and the identification of catalytically active residues through site-directed mutagenesis.

**Results**

**SQR cDNA from *A. marina* is expressed in the yeast mitochondrial membrane**

Screening of recombinant phages in an *A. marina* cDNA library with a heterologous probe for the SQR homolog encoded in the *Drosophila* genome [14] yielded two independent clones of different length. Clone A22-1 contained a full-length cDNA and was 3317 bp long with an ORF of 1377 bp, encoding a protein of 458 amino acid residues (see Fig. 1) with 35% amino acid identity to *S. pombe* SQR (accession no. NP_596067) and 23% amino acid identity to SQR from *R. capsulatus* (accession no. CAA66112).

Expression of the A22-1 ORF in *Escherichia coli* yielded no active SQR enzyme (data not shown); hence, it was cloned into the yeast expression vector pYES2/CT and transformed into INVSc1 yeast cells, whose SQR expression was induced with 20% galactose. SQR was expressed in the mitochondrial membranes of the yeast, as shown by immunodetection of the His tag (Fig. 2). Mitochondria isolated from yeast cells carrying the empty expression vector did not reduce ubiquinone after the addition of sulfide. Using 0.5% Triton X-100, SQR was solubilized from the mitochondrial membranes and purified by nickel nitrilotriacetic acid (Ni-NTA) chromatography. The fractions after purification showed some contaminating proteins (Fig. 2), but, as a result of the low yield and stability of the expressed protein, no further purification steps were applied.

**Cyanide-dependent catalytic properties of recombinant SQR**

The kinetic parameters of *Arenicola* SQR were determined using the pooled and concentrated fractions after Ni-NTA chromatography. It was observed that isolated membranes and isolated SQR were active only in the presence of millimolar concentrations of cyanide, which initially had been introduced to inhibit decyl-ubiquinone$_2$ re-oxidation, but was later found to be required for SQR-dependent decyl-ubiquinone reduction in the absence of thioredoxin and sulfite.
For this reason, 2 mM cyanide was included in the reaction mixture. In the cyanide-dependent reaction, the $K_m$ value for decyl-ubiquinone was 6.4 μM; the $K_m$ value for sulfide of 23 μM was obtained using correction for uncompetitive substrate inhibition, with the corresponding inhibitor concentration yielding half-maximal reaction rate ($K_i$) determined as 480 μM (Fig. 3). The specific activity varied between 1.5 and 5.6 μmol·min$^{-1}$·mg$^{-1}$. Cyanide concentrations up to 20 mM were tested; the $K_m$ value for cyanide was 2.6 mM and the $K_i$ value for substrate inhibition was 0.7 mM (data fitted to the Michaelis–Menten equation corrected for uncompetitive substrate inhibition). The cyanide-dependent SQR reaction had an optimum of pH 9 (Fig. 4). The quinone analog antimycin A inhibited the SQR reaction; the inhibition had a competitive component, as the $K_m$ value for decyl-ubiquinone was elevated to 8 μM in the presence of 10 μM antimycin A, and to 13 μM in the presence of 50 μM antimycin A (Fig. 5).

![Fig. 2. 12% SDS-PAGE after silver staining (lanes 1–5) and western blot analysis with immunodetection of the His tag. Detection was carried out with anti-His IgG (monoclonal mouse IgG, Novagen, Nottingham, UK). Anti-mouse secondary IgG horseradish peroxidase conjugate from goat was used. Ten micrograms of protein were used from fractions of an SQR/His purification from a 4 L culture of Saccharomyces cerevisiae INVSc1 carrying pYES2Ct + SQR. Lane 1, size marker; lane 2, mitochondria; lane 3, mitochondrial membranes; lane 4, SQR/His after one Ni-NTA chromatographic run; lane 5, SQR/His after two Ni-NTA chromatographic runs; lane 6, post-mitochondrial supernatant; lane 7, mitochondria; lane 8, soluble mitochondrial proteins; lane 9, mitochondrial membranes; lane 10, SQR/His after Ni-NTA chromatography. Arrows indicate the SQR/His bands at 50 kDa.](image1)

![Fig. 3. Affinity of SQR/His for sulfide in the presence of cyanide or thioredoxin. Left: Michaelis–Menten plot corrected for uncompetitive substrate inhibition for sulfide affinity of SQR/His in the presence of cyanide ($K_m = 22.9$ μM; $K_i = 480$ μM; $V_{max} = 5.3$ μmol·min$^{-1}$·mg$^{-1}$). Right: Michaelis–Menten plot corrected for uncompetitive substrate inhibition for sulfide affinity of SQR/His in the presence of thioredoxin and sulfite ($K_m = 23.3$ μM; $K_i = 3.8$ μM; $V_{max} = 0.66$ μmol·min$^{-1}$·mg$^{-1}$). For plotting, the Enzyme Kinetics Module of the program SIGMA PLOT 9.0 (Jandel Scientific, San Rafael, CA, USA) was used. $n = 3$.](image2)
The product of the SQR reaction in the presence of cyanide is not thiosulfate, but thiocyanate

Thiosulfate and sulfite were not detected in greater amounts in assay mixtures with SQR than in control mixtures without enzyme. However, thiocyanate was detected as a product of the reaction. In the presence of 100 μM decyl-ubiquinone, 43 ± 7 nmol thiocyanate was detected after 65 min. In the presence of 200 μM decyl-ubiquinone, the concentration of thiocyanate increased to 60 ± 5 nmol after 5 min of incubation.

**Arenicola SQR shows a thioredoxin-dependent activity**

Cyanide has been described as an *in vitro* substrate for rhodanese (E.C. 2.8.1.1) [23,24]. Rhodanese is also active if thioredoxin is used instead of cyanide [25,26]. Therefore, we tested thioredoxin as a cosubstrate for *Arenicola* SQR in the presence of 15 μM thioredoxin (reduced by thioredoxin reductase) and millimolar concentrations of sulfite. Sulfite was introduced because *Arenicola* mitochondria are known to produce thiosulfate from sulfide [7]. The *Kₘ* value for sulfide in the presence of thioredoxin and sulfite was 23 μM with a *Vₘₐₓ* value of 0.66 μmol·min⁻¹·mg⁻¹. The *Kᵢ* value for substrate inhibition was 3.8 μM.

**Three SQR fingerprints were found in Arenicola SQR**

In eukaryotic SQR sequences, three of five SQR fingerprints identified by Griesbeck *et al.* [9] were conserved [14]. These fingerprints were also found in *Arenicola* SQR. Phylogenetic analysis of SQR sequences revealed three groups of sequence diversity [13], with group II representing all eukaryotic sequences. *Arenicola* SQR is a member of this group (data not shown).

**Site-directed mutagenesis of six conserved amino acids in eukaryotic SQRs leads to a loss of activity for each mutated protein**

In separate constructs, the two cysteine residues Cys208 and Cys386 were replaced with serine, the histidine residues His86 and His299, and Glu159, with alanine, and Asp342 with valine. All mutated proteins were expressed in the mitochondrial membrane of yeast, but none of the proteins showed detectable activity, in contrast with the A22-1 control.

**Discussion**

The first eukaryotic SQR was described for the fission yeast *S. pombe* [27]. As the *Kₘ* values of the enzyme for sulfide and quinone were in the millimolar range, the *in vivo* function as an SQR remained contentious. Recently, many homologs of *S. pombe* SQR have been identified in other eukaryotic genomes [14], but none of these has previously shown catalytic activity. Sulfide-detoxifying enzymes are essential for animals, such as the lugworm *A. marina*, that are often exposed to high sulfide concentrations in their habitats. Little is yet known about the enzymes involved in mitochondrial sulfide oxidation, but biochemical evidence has
been reported for an SQR in the mitochondria of lugworms [5,7].

The SQR from Arenicola is catalytically active in the presence of cyanide

The enzyme was expressed in yeast mitochondrial membranes and purified using Ni-NTA affinity chromatography. Decyl-ubiquinone was reduced after the addition of sulfide, but only in the presence of cyanide. This was surprising, because bacterial SQR requires no additional substrate other than sulfide and quinone, and, for the SQR from S. pombe, cyanide-independent activity has been described [27]. However, the $K_m$ values for sulfide and quinone of 2 mM reported for S. pombe SQR were orders of magnitude higher than those reported for bacterial SQR, whose $K_m$ values were in the range 2–8 μM (Table 1); accordingly, the in vivo role of S. pombe SQR as a sulfide-oxidizing enzyme was called into question [9]. In this study, we aimed to characterize an SQR from a eukaryote, A. marina, that encounters physiologically relevant concentrations of sulfide in its natural environment. Initially, cyanide was included in the reaction mixture when intact mitochondria were measured to inhibit cytochrome c oxidase and thus to avoid a re-oxidation of ubiquinone. However, it was found that cyanide is a cosubstrate for purified SQR with a $K_m$ value of 2.6 mM. These findings are supported by the recent report of a cyanide-dependent increase in SQR activity for the enzyme from Pseudomonas putida [28], which, like A. marina SQR, belongs to the sequence group II designated previously [14].

The end product of the cyanide-dependent reaction is thiocyanate. The spectrophotometric detection of thiocyanate is a general method for the quantification of sulfane sulfur [29], as first described for rhodanese [30,31], which catalyzes the sulfur transfer from thiosulfate to cyanide with the formation of thiocyanate. However, it was found that cyanide is a cosubstrate for purified SQR with a $K_m$ value of 2.6 mM. These findings are supported by the recent report of a cyanide-dependent increase in SQR activity for the enzyme from Pseudomonas putida [28], which, like A. marina SQR, belongs to the sequence group II designated previously [14].

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Table 1. Comparison of mean $K_m$ values for sulfide, decyl-ubiquinone and cyanide of Arenicola marina, Schizosaccharomyces pombe [26] and Rhodobacter capsulatus [9] SQR.

<table>
<thead>
<tr>
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<th>$K_m$ sulfide</th>
<th>$K_m$ ubiquinone</th>
<th>$K_m$ cyanide</th>
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<tbody>
<tr>
<td>A. marina</td>
<td>23 μM</td>
<td>6.4 μM</td>
<td>2.6 mM</td>
</tr>
<tr>
<td>S. pombe</td>
<td>2 mM</td>
<td>2 mM</td>
<td>Not determined</td>
</tr>
<tr>
<td>R. capsulatus</td>
<td>2 μM</td>
<td>2 μM</td>
<td>Inhibitor</td>
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Arenicola SQR interacts with thioredoxin

Cyanide is not usually produced endogenously in large amounts by animals, and millimolar concentrations cannot be found in the environment. Thus, cyanide is probably not the in vivo cosubstrate of SQR. Thioredoxin was tested for its interaction with SQR. SQR was active in the presence of thioredoxin, but only if sulfite was added to the reaction mixture. This suggests a more complex sulfide detoxification pathway, involving at least one more enzyme in addition to SQR.

SQR does not produce thiosulfate, it is a persulfide donor

For bacterial SQR, the reaction mechanism has been described [9]. Three conserved cysteines play an essential role in the reductive half-reaction. As eukaryotic SQR lacks a third cysteine [14], the cysteine-bound persulfide must be transferred to an external acceptor to enable the electron transfer on FAD. This suggests a function of SQR as a persulfide donor. Indeed, there have been reports of sulfane sulfur formation in the sipunclid Phascolosoma arcuatum [32] and the mudskipper Boleophthalmus boddartii [33] under sulfidic and anaerobic conditions. A possible mechanism of persulfide formation is shown in Fig. 6, involving Cys208 and Cys387. Glu159 may play a role as the active site base, in analogy with the bacterial reaction [9]. The oxidative half-reaction of Arenicola SQR may be similar to the proposed bacterial oxidative reaction, involving two histidines for acid–base catalysis [9].

Asp342 is required for Arenicola SQR function

The mutation of Asp342 to valine led to an inactive SQR enzyme. The FAD-binding domain of all eukaryotic SQRs, including A. marina SQR, contains a conserved aspartate at position 342 (numbering according to the Arenicola sequence; marked in bold in Fig. 1). This is in contrast with bacterial SQRs, which possess valine at this position [9,14]. Griesbeck et al. [9] showed that an exchange of Val300 to Asp300 in Rhodobacter SQR reduced the activity to 11% of wild-type activity. Changing the corresponding residues, Asp342 to Val342, in Arenicola SQR function

The exchange of Asp342 to Val342 in Arenicola SQR
might affect FAD binding, although a loss of SQR activity as a result of misfolding of the mutant enzyme cannot currently be excluded.

The physiological role of mitochondrial SQR in lugworms and higher eukaryotes

Animals inhabiting sulfide-rich environments require powerful mechanisms to detoxify sulfide. However, SQR homologs can be found in most, but not all, animal genomes. As a relict of the sulfidic and anoxic phase of the Earth’s history, when all marine organisms had to deal with high environmental sulfide concentrations [15–17], SQR might have played a role. In eukaryotes that do not today inhabit sulfidic environments, sulfide has been discussed as a modulator of physiological responses and an atypical neuromodulator, in addition to the gases NO and CO [37]. Endogenous sulfide production has been described, not only for marine invertebrates, such as *A. marina* and the mussel *Tapes philippinarum* [21], that deal with high environmental concentrations of sulfide daily, but also for various mammals that do not [18–20].

Starting from L-cysteine, endogenous sulfide can be synthesized in at least four different ways [38]. In mitochondria, cysteine-aminotransferase (E.C. 2.6.1.3) and 3-mercaptop-sulfurtransferase (E.C. 2.8.1.2) can be involved in sulfide production [38]. Cysteine-aminotransferase catalyzes the reaction of L-cysteine with a ketoacid (e.g. α-ketoglutarate), with the formation of 3-mercaptoppyruvate and an amino acid (e.g. L-glutamate). 3-Mercaptoppyruvate is desulfurized by 3-mercaptoppyruvate-sulfurtransferase, resulting in the formation of sulfide and pyruvate [21]. In the cytosol, sulfide can be generated by cystathione-β-synthase (E.C. 4.2.1.22). Alongside endogenous sulfide production in mammals, considerable amounts of sulfide can be produced by anaerobic sulfate-reducing bacteria in the human colon, posing a challenge to cells of the intestinal epithelium [39].

Such findings suggest that even animals that are not exposed to environmental sulfide require biochemical means of dealing with sulfide, albeit at lower concentrations than those experienced by sulfide-exposed marine invertebrates. A failure to deal with endogenous sulfide can have dire consequences in humans. For example, the overproduction of sulfide as a result of enhanced cystathione-β-synthase activity can exacerbate cognitive effects in Down’s syndrome patients [22,40], and insufficient detoxification of sulfide produced in the human colon can lead to inflammatory diseases and may affect the frequency of colon cancer [41]. Whether or not SQR plays a significant physiological role in mammalian sulfide metabolism remains to be shown.

Materials and methods

Yeast growth conditions

INVSc1 cells (Invitrogen, Carlsbad, CA, USA) were grown at 30 °C in SC minimal medium (0.67% yeast nitrogen base without amino acids, 2% glucose, drop-out medium without uracil). Protein expression was induced by replacing glucose with galactose (2%) and raffinose (1%).
RNA isolation and cDNA synthesis

RNA was isolated from approximately 10 g of body wall tissue of *A. marina* collected from the Dutch coast. For mRNA isolation, the mRNA Purification Kit (GE Healthcare Biosciences, Uppsala, Sweden) was used. cDNA was synthesized using the Time Saver cDNA Synthesis Kit (GE Healthcare Biosciences). Total RNA from *Drosophila* was isolated using the Nucleospin RNA II Kit (Macherey-Nagel, Dueren, Germany). For cDNA synthesis, the first-strand synthesis kit for RT-PCR (Invitrogen) was used.

Hybridization probe, cloning and heterologous expression

Standard molecular and biochemical methods, cDNA synthesis and cloning in *S. cerevisiae* were performed as described previously [42]. *Drosophila melanogaster* SQR (NP_647877) was amplified using 5'-ATGAACCGCTCGTCTTCAGG AACC-3' and 5'-GCACCTGACAAAAATTTTTCGCATT AGTGGC-3' as primers. DNA was sequenced by the Sanger dideoxy method [43]. For heterologous expression of *A. marina* SQR in *S. cerevisiae*, the shuttle vector pYES2/CT (Invitrogen) with a C-terminal His tag was used. SQR was cloned into the *HindIII/XbaI* site.

Site-directed mutagenesis

The following primers were designed using the program ‘THE PRIMER GENERATOR’ (http://www.med.jhu.edu/medcenter/primer/primer.cgi [44]): Asp342Val, 5'-GTCTTCCGCTC GTGTTCAACACGGATATACCG AACC-3' and 5'-GCACCTGACAAAAATTTTTCGCATT AGTGGC-3'; Cys208Ser, 5'-GCCCATCAAAATCGTCAGCGCGCCGCGC-3' and 5'-CGGGTAGTTTA CGGCTACACGTC CCGCGCGGCG-5'; Cys386Ser, 5'-CGGCTGATCTCTTCCCCCTGTTGACG-3' and 3'-GCCGATGTGCAG CGGACAC-5'; His86Ala, 5'-GCCGACAC-3' and 3'-CAGAAGC CCGTGGTGCCT-3'; His299Ala, 5'-GCCATTCGATCATGAGGC-5'; His399Ala, 5'-GCCATGCGGTGGAATGCT-3' and 3'-CGGTAC GACCCGCACCACCGGA-5'; Glu59Ala, 5'-GGGCTGCC TGCAGCTTCC-3' and 3'-CCCCACGCGAGTCCGGAAG-5'; nucleotides modified from the wild-type sequence are shown italic. PCR was performed as described previously [45]. Mutated SQRs were cloned into pYES2/CT and expressed in INVSc1.

Isolation of yeast mitochondria

*S. cerevisiae* carrying pYES2/CT + SQR was grown at 30 °C for 24 h. The cells were harvested by centrifugation (5 min, 1000 *g*) at 20 °C. The cells were washed with H2O, followed by a washing step with washing buffer (20 mM Tris/HCl, pH 7.4, 50 mM NaCl, 0.6 M sorbitol). The cell pellet was resuspended in 30 mL of washing buffer containing Yeast/Fungal Protease Inhibitor Cocktail (Sigma, St Louis, MO, USA), and incubated on ice for 5 min. The cells were broken by rigorous vortexing for 3 × 1 min at 4 °C. Unbroken cells and cell debris were centrifuged at 800 *g* at 4 °C for 5 min. The supernatant was centrifuged for 20 min at 10 000 *g* at 4 °C. The pellet (mitochondria) was resuspended in 20 mL of washing buffer containing protease inhibitor.

Purification of SQR/His

Isolated mitochondria were broken by sonication. Membranes were isolated by 1 h of ultracentrifugation at 30 000 r.p.m. (Sorvall Ultra Pro 80, rotor T-865). The pellet was resuspended in 5 mL of solubilization buffer (50 mM NaP, pH 7.2, 5% glycerol, 320 mM NaCl, 0.5% Triton X-100) and stirred on ice for 1 h. The suspension was loaded on a 1 mL Ni-NTA (Qiagen, Hilden, Germany) column, and SQR was eluted with an imidazole gradient using an FPLC system (GE Healthcare Biosciences). Fractions containing activity were pooled and concentrated to 1 mL using Amicon Ultra-15 centrifugal filter devices (Millipore, Billerica, MA, USA).

SQR activity assay

In the cyanide-dependent activity assay, SQR activity was measured under air at room temperature. A 1 mL reaction contained 20 mM Tris/HCl, pH 8.0, 100 μM decyl-ubiquinone (Sigma), 2 mM KCN and either isolated mitochondria, membranes or purified enzyme. The reaction was started with 200 μM sulfide (prepared freshly with N2-flushed H2O) and the decrease in absorption at 275 nm was followed for 3 min (modified from [27] and [46]). An extinction coefficient of 15 L·mmol⁻¹·cm⁻¹ for decyl-ubiquinone was used [47].

In the thioredoxin-dependent activity assay, a 1-mL reaction contained 50 mM potassium phosphate, pH 8.2, 100 μM decyl-ubiquinone, 20 mM sulfite (prepared freshly with N2-flushed H2O), 15 μM thioredoxin (from *E. coli*, Sigma), 0.2 U thioredoxin-reductase (from *E. coli*, Sigma), 1 mM NADPH and either isolated mitochondria, membranes or purified enzyme. The reaction was started with sulfide and the decrease in absorption at 275 nm was followed for 5–10 min.

Determination of pH optimum and inhibition studies

The pH optimum of the cyanide-dependent SQR reaction was determined using sodium phosphate, Tris, Caps, Bicine, and Hepes, covering a pH range from 5.8 to 11.1. Measurements were carried out at 22.5 °C. Antimycin A (Sigma) was used for inhibition studies at 10 and 50 μM.
Determination of end products

Sulfide, sulfate and thiosulfate were determined by HPLC using the bromobimane method modified from [48] and [49]. Thiocyanate was determined as described previously [23,30,31].

Phylogenetic network and fingerprint analysis

A phylogenetic network and fingerprint analysis of SQR sequences, including Arenicola SQR, was performed as described previously [14].

Determination of kinetic constants

Kinetic parameters were determined using nonlinear least-square analysis of the data fitted to the Michaelis–Menten rate equation \( v = V_{\text{max}} \frac{[S]}{K_m + [S]} \) or, where indicated, the Michaelis–Menten equation corrected for uncompetitive substrate inhibition \( v = V_{\text{max}} \frac{[S]}{K_m + [S](1 + [S]/K_i)} \), where \( v \) is the velocity, \( V_{\text{max}} \) is the maximum velocity, \( S \) is the substrate concentration, \( K_m \) is the Michaelis–Menten constant and \( K_i \) is the inhibition constant, using SIGMA-Plot 9.0 (Systat Software, Erkrath, Germany) and the enzyme kinetic module 2.0.

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