High Resolution X-ray Crystallography Shows That Ascorbate Is a Cofactor for Myrosinase and Substitutes for the Function of the Catalytic Base

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Myrosinase, an S-glycosidase, hydrolyzes plant anionic 1-thio-β-D-glucosides (glucosinolates) considered part of the plant defense system. Although O-glycosidases are ubiquitous, myrosinase is the only known S-glycosidase. Its active site is very similar to that of retaining O-glycosidases, but one of the catalytic residues in O-glycosidases, a carboxylate residue functioning as the general base, is replaced by a glutamine residue. Myrosinase is strongly activated by ascorbic acid. Several binary and ternary complexes of myrosinase with different transition state analogues and ascorbic acid have been analyzed at high resolution by x-ray crystallography along with a 2-deoxy-2-fluoro-glucosyl enzyme intermediate. One of the inhibitors, d-glucono-1,5-lactam, binds simultaneously with a sulfate ion to form a mimic of the enzyme-substrate complex. Ascorbate binds to a site distinct from the glucose binding site but overlapping with the aglycon binding site, suggesting that activation occurs at the second step of catalysis, i.e. hydrolysis of the glycosyl enzyme. A water molecule is placed perfectly for activation by ascorbate and for nucleophilic attack on the covalently trapped 2-fluoro-glucosyl-moiety. Activation of the hydrolysis of the glucosyl enzyme intermediate is further evidenced by the observation that ascorbate enhances the rate of reactivation of the 2-fluoro-glycosyl enzyme, leading to the conclusion that ascorbic acid substitutes for the catalytic base in myrosinase. Glucosinolates are anionic β-D-S-glucosides found prominently in plants of the genus Brassica (cabbage, mustard, rapeseed, and other Cruciferae). They constitute a large family of S-glucosides that differ by their aglycon (Ref. 1 and Fig. 1a). The same plants produce myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1), an S-glycosidase hydrolyzing glucosinolates. Myrosinase and glucosinolates are stored in different compartments of the plant, especially in the seeds. Mixing of enzyme and substrate (for example through mastication) induces glucosinolate hydrolysis. The biological function of myrosinase and glucosinolates is only partly elucidated; it has been suggested that they represent a defense system of the plant. Glucosinolates may as well serve to store inactive precursors of hormones such as 3-indolylacetic acid. 3-Indolyl acetonitrile and related indoles (2) are released from indolyl glucosinolates by myrosinase (3). Cleavage of indol-3-ylmethyl glucosinolate (glucobrassicin) by myrosinase in the presence of ascorbic acid produces ascorbigen, a condensation product of ascorbic acid with 3-hydroxymethylindole (4). Thus, the myrosinase system could be involved in the storage and in the inactivation of ascorbic acid. A detailed review on myrosinase is given by Bones and Rossiter (3).

Myrosinase hydrolyzes the S-glycosides with retention of the anomeric configuration (5). Retaining glycosidases operate by a double displacement at the anomeric center promoted by two carboxylic residues acting as acid/base and as nucleophile, respectively. In the first step (glycosylation), the glycosidic oxygen is protonated by the carboxyl group acting as general acid, whereas the second catalytic residue, a carboxylate group, performs a nucleophilic attack at the anomeric carbon. A glycosyl enzyme intermediate with inverted anomeric configuration is formed via a transition state featuring a (more or less) planar (trigonal) anomeric carbon. In the second step (deglycosylation), the carboxylate group corresponding to the catalytic acid residue activates a water molecule that attacks at C-1 of the glycosyl enzyme to yield the hemiacetal and the free enzyme, again via a transition state with a trigonal anomeric carbon (6).

Many carbohydrate derivatives with a planar anomeric carbon and a half-chair or a distorted half-chair conformation inhibit glycosidases. Three such inhibitors, d-glucono-1,5-lactone (Refs. 7 and 8 and Fig. 1c), nojirimotezol (Refs. 9 and 10 and Fig. 1d), and d-gluconohydrin-1,5-lactam (Ref. 11 and Fig. 1e), have been studied. A detailed review is given by Heightman and Vasella (12).

Myrosinase belongs to family 1 of the glycoside hydrolases (13–15) but is an unusual member of this family in that it lacks the acid/base residue in its active site. Although classical glycosidases activate the glycosidic oxygen by the catalytic acid residue in the glycosylation step, no such activation appears necessary nor indeed possible for myrosinase. Once the glycosyl enzyme intermediate is formed, the glutamine residue that replaces the catalytic glutamate residue of the classical β-glycosidases ensures the correct positioning of a water molecule, without deprotonating it. This positioning is sufficient to allow hydrolysis of the glycosyl enzyme intermediate and the release of the products (16).
The covalent glycosyl enzyme intermediate of β-glycosidases can be trapped using substrates that are fluorinated at C-2 (i.e., adjacent to the scissile glycosidic bond) and that carry an aglycon with good leaving group ability (17, 18) (Fig. 1b). This approach has made it possible to observe several relatively stable 2-fluoro-glycosyl enzyme intermediates by x-ray crystallography (16, 19–21) (Fig. 1f).

Even though myrosinase shows considerable activity in absence of L-ascorbic acid, its activity is enhanced by ascorbate, as first described by Nagashima and Uchiyama (22). The physiological significance of this activation stems from an optimal effect by L-ascorbate compared with its derivatives (23), suggesting that the enzyme has evolved to work with the naturally occurring L-ascorbate. A physiological role of the activation by ascorbate is further evidenced by the ascorbate concentration needed for optimal activation of about 1.5 mM, in the range of the global ascorbate concentration in the concerned plant tissues, e.g., 2 mM in horseradish root (24). Ascorbic acid is stored in the vacuoles of plant cells (25) where the local concentration is much higher (24).

A 25-fold increase of the enzymatic activity of Brassica juncea seed myrosinase on the natural substrate sinigrin upon addition of ascorbate, using sinigrin as the substrate. Variable degrees of activation, ranging from 1.8- to 11-fold depending on the source of myrosinase (Brassica napus, Brassica campestris, and S. alba), the isoenzyme used, and the substrate have been reported by Björkman and LönnérDAL (28). These authors also found an increase in $K_m$ upon activation by ascorbate and concluded that the stability of the enzyme-substrate complex was reduced, presumably because of an increase in the rate constant for the formation of the product. More recently, Shikita et al. (29) described a 140-fold increase in $V_{max}$ in the presence of 0.5 mM ascorbate for the cleavage of sinigrin by myrosinase from Raphanus sativus. This increase in $V_{max}$ was paralleled by an increase of $K_m$, an observation that has been interpreted as the result of a noncompetitive activation, because of binding of ascorbate to the enzyme-substrate complex. All the authors quoted above noticed the dual behavior of ascorbate, which activates myrosinase at 0.1–1.0 mM and acts as a competitive inhibitor at higher concentrations, typically above 1.5 mM.

Ettlinger et al. (23) have studied the specificity of myrosinase activation by different derivatives of L-ascorbate, concluding that an acidic group is essential for the activation and that activation is independent of the reducing property of ascorbate. These investigators suggested that ascorbate plays the role of the catalytic acid/base.
We have initiated a program to analyze the details of the mechanism of action of myrosinase and the effect of L-ascorbate using a myrosinase from white mustard (S. alba) grains. This enzyme is a dimer with a mass of 130 kDa, of which 30 kDa are due to glycosylation (16). A detailed view on the enzymatic mechanism of myrosinase is derived from high resolution (1.2–1.6 Å) x-ray crystal structure analysis of binary and ternary complexes of myrosinase with inhibitors mimicking the transition state, with ascorbic acid, and with the stable 2-fluoro-glucosyl enzyme intermediate.

MATERIALS AND METHODS

Reagents—2-F-GTL* (Fig. 1b) was synthesized as described previously (30). d-Glucono-1,5-lactone (Fig. 1c) and ascorbic acid (Fig. 1g) were purchased from Sigma. Gluco-tetrazole (Fig. 1d) and gluco-hydroximolactam (Fig. 1e) were synthesized as described (9, 11). C-GTL, the C-glycosidic analogue of glucotropaeolin (Fig. 1h), was synthesized as a mixture of Z- and E- stereoisomers as described earlier (31).

Data Collection—Myrosinase crystals were prepared as described (16) using ammonium sulfate as a precipitant. The purified myrosinase remained suitable for crystallization after storage for 3 years at 4 °C in 20 mM HEPES buffer, pH 6.5. For cryoprotection, a crystal was transferred to a solution containing 86% (v/v) saturated ammonium sulfate, 100 mM HEPES, pH 6.5, and 10% (v/v) glycerol. Before freezing in a nitrogen stream at 100 K, the crystal was dipped for a few seconds into a solution containing 20% glycerol. For some trials, glycerol was replaced by ethylene glycol. The inhibitors were bound by soaking crystals in artificial mother liquor containing the compound. To obtain the 2-fluoro-glucosyl enzyme, the native crystals were soaked overnight with 2.5 mM 2-F-GTL as the hydrolysis of this compound is slow. For the ternary complex with 2-F-GTL and ascorbate, the ascorbate was introduced subsequently in the cryoprotectant containing 10% glycerol. Data were collected at the European Synchrotron Radiation Facility (Grenoble, France) on experimental station ID14-3 using a 133-mm MarCCD detector or on ID14-1 using a Mar345 detector (gluconolactone data set). Data were processed with MOSFLM (32) and the CCP4 package (33).

Refinement—Statistics of data collection and refinements are given in Table I. The initial model was entry 1MYR deposited in the Protein Data Bank (16). The native structure has been refined using first X-PLOR 3.1 (34) and then REFMAC (35) to a final resolution of 1.2 Å. During the refinement a number of changes in the sequence, which was based on an x-ray structure at 1.6 Å resolution, became apparent and have been corrected. The complexes have been analyzed with SIGMAA weighted 

\[ F_{\text{calc}} - F_{\text{obs}} \] maps calculated with model phases. The model is based on the native high resolution structure from which the water and glycerol molecules in the active site have been removed. A direct interpretation of a 

\[ F_{\text{obs}} - F_{\text{calc}} \] Fourier synthesis is hampered by the presence of a glycerol molecule and several well ordered water molecules in the active site. The structure of the complexes have been refined as described for the native structure. Coordinates and structure factors have been deposited in the Protein Data Bank (see Table I for accession numbers).

Enzyme Kinetics—Time-dependent reactivation of the 2-fluoro-glucosyl enzyme catalytic intermediate in absence or in presence of ascorbic acid was determined essentially as described previously (5). Myrosinase was inactivated with 2.5 mM 2-F-GTL (30) for 18 h at 40 °C, followed by four successive ultrafiltrations on Nanosep 30 kDa (Pall Filtron Corp.) to remove the excess of inactivator. The inactivated enzyme was incubated at 25 °C without or with ascorbic acid (at concentrations of 0.1 and 1 mM), and reactivation was monitored using aliquots (20 μl) from the solutions of inactivated enzyme at appropriate time intervals. The samples were assayed by using p-nitrophenyl-β-D-glucopyranoside (36) as the substrate, following the variation of absorbance at 490 mM (A = 7002 M−1 cm−1 for 4-nitrophenol). First-order rate constants (k_recov) were determined by fitting the recovered activity as a function of time.

The inhibition constants for gluco-tetrazole and gluco-hydroximolactam have been determined with sinigrin as substrate. The hexokinase/glucose-6-phosphate dehydrogenase coupled enzyme system was used to assay myrosinase activity by measuring glucose release, following the variation of absorbance at 340 nm (37). Myrosinase was preincubated at 34 °C (10 μl at appropriate dilution) in the absence or presence of inhibitor (38 μl at final concentrations of 0.4, 0.8, and 2 mM for the tetrazole and 0.125, 0.5, and 1 mM for the hydroximolactam). Reactions were initiated by addition of substrate (332 μl at final concentration of 0.17, 0.24, 0.35, and 0.7 mM in 50 mM Mes buffer, pH 6.5, containing 3 mM MgCl2, 0.55 mM ATP, 0.72 mM NADP, 0.56 unit/ml hexokinase, and 0.35 unit/ml glucose-6-phosphate dehydrogenase). Kinetic data were fitted to a competitive inhibition model (38). All reagents were purchased from Sigma.

RESULTS

Three established or putative transition state analogues, gluco-tetrazole, d-glucono-1,5-lactone, and gluco-hydroximolactam were soaked into the crystals. The resulting electron densities (Fig. 2, a–c) show that the three inhibitors bind similarly, with all hydroxyl groups involved in identical hydrogen bonds. This recognition is similar to that observed for the 2-fluoro-glycosyl enzyme intermediate (Fig. 2d), except that the three inhibitors display a somewhat distorted half-chair conformation, whereas the glucose ring in the 2-fluoro-glycosyl enzyme has a clear C-terminal chair conformation (Fig. 3).

d-Glucono-1,5-lactone binds in a distorted half-chair conformation (Fig. 3) with O-1 of the lactone in van der Waals’ contact with Oe1 of Gln 187 (Fig. 2f). The binding of d-glucono-1,5-lactone with full occupancy at a concentration of 20 mM in the active site at the exact position of the glucose moiety of the substrate is in contradiction with the experiments by Botti et al. (36), who concluded that the d-glucono-1,5-lactone is a non-competitive inhibitor with a Ki of 5 mM.

The inhibition constants for gluco-tetrazole (Ki = 0.7 mM) and gluco-hydroximolactam (Ki = 0.6 mM) were determined using sinigrin as substrate (data not shown). The gluco-tetrazole binds in a pure half-chair conformation (Figs. 2b and 3). There is a hydrogen bond from Gln 187 Oe1 to N1 of the tetrazole for which there is no equivalent for the gluconolactone inhibitor. This hydrogen bond is in the plane of the tetrazole moiety and may explain why the gluco-tetrazole inhibitor is bound at a slightly higher position in the active site than the other inhibitors. It is noteworthy that the residue equivalent to myrosinase Glu 187 in related O-glucosidases, such as the cyanogenic β-glucosidase from white clover (39), is the catalytic acid/base glutamate that would be in an appropriate position for an “in plane” protonation of the substrate as predicted by Heightman and Vasella (12).

The structure of the complex with gluco-hydroximolactam shows a slightly distorted half-chair conformation for the inhibitor (Fig. 3). In addition to the inhibitor, a sulfate ion was found bound in the active site. This sulfate ion forms salt bridges and hydrogen bonds to Arg259 Nε2, Gln 187 Nε2, and the hydroxyl group of the hydroximoo group (Fig. 2c). The N1 nitrogen atom of this group is in van der Waals’ contact with the oxygen Oε1 of the carbonyl group of Gln 187.

Several, albeit unsuccessful, experiments have been undertaken to observe substrate binding directly. The diastereomeric mixture of C-GTL (Fig. 1a) did not inhibit at concentrations up to 20 mM (enzyme assays; data not shown), nor did it bind in the crystal at concentrations of up to 100 mM. We also attempted to take advantage of the slow hydrolysis of 2-F-GTL by myrosinase and to trap the enzyme-substrate complex using flash freezing of the crystals. Cottaz et al. (5) determined inactivation parameters Ks of 0.9 mM and k of 0.083 min−1 for 2-F-GTL. These data suggest that it should be possible to observe the enzyme-substrate complex. A number of experiments using different soaking times (5–20 min) and different pH values of the buffer (pH 4.2–6.5) did not lead to the observation of any electron density corresponding to the substrate analogue. The nonglucosylation of Glu459 confirms that myrosinase is only very slowly inactivated by 2-F-GTL.

*The abbreviations used are: 2-F-GTL, 2-F-glucotropaeolin; Mes, 4-morpholinoethanesulfonic acid.
### Table I

| Data set          | Native | Ascorbate | Ascorbic acid (10 mM) | Glucohydroximolactam (5 mM) | Glucohydroximolactam (20 mM) |
|-------------------|--------|-----------|-----------------------|-----------------------------|-----------------------------|
| Glucocetramide    |        |           |                       |                             |                             |
| 2-F-GTL (2.5 mM)  | 151092 | 90017     | 110239                | 84662                       | 98776                       |
| Redundancy (%)    | 4.5    | 3.1       | 2.7                   | 3.3                         | 2.4                          |
| Complete (%)      | 99.6   | 95.8      | 83.7                  | 97.1                        | 88.5                        |
| Rmerge (%)        | 7.6    | 7.2       | 7.5                   | 8.9                         | 8.5                          |
| Rcryst (%)        | 12.4   | 11.9      | 12.0                  | 13.8                        | 16.9                         |
| Rfree (%)         | 14.2   | 14.6      | 15.2                  | 17.8                        | 19.5                         |
| Average bond length (Å) |       |           |                       |                             |                             |
|                   | 0.015  | 0.017     | 0.019                 | 0.018                       | 0.016                       |

### Myrosinase Reaction Mechanism

To study the intriguing activation of myrosinase by ascorbate, myrosinase crystals were soaked with ascorbate alone. Omit maps showed clearly the presence of ascorbate together with a glycerol molecule in the active site (Fig. 4a). Ascorbate is recognized by a salt bridge between Arg$^{259}$ Ne and the O-1 oxygen and by a hydrogen bond between the hydroxyl group at position 2 and Arg$^{259}$ Nq2. O-3 forms a hydrogen bond with Ne2 of Gln$^{187}$ (Fig. 4a). A portion of the ascorbic acid molecule is located in the hydrophobic pocket, which binds the hydrophobic part of the glucosinolates. This pocket is formed by the residues Ile$^{257}$, Phe$^{331}$, Tyr$^{330}$, Phe$^{371}$, and Phe$^{473}$, which are invariant in the nine known myrosinase sequences with exception of Phe$^{331}$. Another conserved residue, Arg$^{194}$ (Lys in one sequence), is not directly in contact but may play an electrostatic role. The binding of ascorbate is remarkably similar to the binding of the sulfate ion in the gluco-hydroximolactam inhibitor. A soak with a mixture of gluco-hydroximolactam and ascorbic acid showed that there is indeed competition between the binding of sulfate and ascorbate. The structure shows a partial occupancy for ascorbate ($q = 0.6$) and for sulfate ($q = 0.4$) (Fig. 4b). Two of the oxygen atoms of the sulfate ion are in positions similar to O-2 and O-3 of ascorbate, interacting with Ne2 of Gln$^{187}$ and Arg$^{259}$ Nq2.

From their common position, it is obvious that ascorbate and the intact substrate cannot bind together in the active site. However, the presence of both ascorbate and gluco-hydroximolactam (Fig. 4b) or of ascorbate in the 2-fluoro-glucosyl enzyme (Fig. 4c) shows that ascorbate can bind once the aglycon of the substrate has diffused away. In the presence of the 2-fluoro-glucosyl group or of the gluco-hydroximolactam, a hydrogen bond is formed between the O-6 hydroxyl group of glucose and the O-6 hydroxyl group of ascorbic acid (Fig. 4, b and c). Ascorbate is placed ideally to act as a catalytic base and to activate the O-6 hydroxyl group of ascorbic acid (Fig. 4, b and c). Additionally, the presence of gluco-hydroximolactam inhibits the binding of the sulfate ion in the gluco-hydroximolactam inhibitor. The binding of ascorbate is remarkably similar to the binding of the sulfate ion in the gluco-hydroximolactam inhibitor. A soak with a mixture of gluco-hydroximolactam and ascorbic acid showed that there is indeed competition between the binding of sulfate and ascorbate. The structure shows a partial occupancy for ascorbate ($q = 0.6$) and for sulfate ($q = 0.4$) (Fig. 4b). Two of the oxygen atoms of the sulfate ion are in positions similar to O-2 and O-3 of ascorbate, interacting with Ne2 of Gln$^{187}$ and Arg$^{259}$ Nq2.

The activation of the hydrolysis of the glucosyl enzyme by ascorbate has been verified using the 2-F-GTL-inactivated enzyme. In the absence of ascorbate, half-reactivation required 53 h. Addition of 1 mM ascorbate resulted in a 14-fold activation, shortening the half-reactivation time to 3.6 h (Fig. 5) and demonstrating that activation concerns hydrolysis of the glucosyl enzyme.

### DISCUSSION

The good affinity displayed by the transition state analogous inhibitors (gluco-tetrazole and gluco-hydroximolactam) is in agreement with the hypothesis that the reaction mechanism catalyzed by myrosinase is similar to the one for the related $p$-O-glucosidases (12). The position of the sulfate ion in the gluco-hydroximolactam complex is close to the one suggested for the sulfate group of glucosinolates, as modeled in a putative enzyme-substrate complex (16). It is possible to model a sinigrin molecule using the position of the gluco-hydroximolactam inhibitor and of the sulfate ion and to visualize the enzyme-substrate complex (Fig. 6). The position of the sulfate group and of the glucose moiety determines the position of the hydrophobic moiety of the aglycon that is expected to bind in the hydrophobic pocket formed by the three phenylalanine resi-
dues Phe$_{331}$, Phe$_{371}$, and Phe$_{473}$, with residue Tyr$_{330}$ at the bottom. It should be noted that the substrate has to adopt a distorted boat conformation similar to that described by Davies et al. (19) to avoid a steric clash with amino acid side chains. According to modelization, the sulfate group of the glucosinolate is bound to Arg$_{259}$ N$_{h2}$ and Gln$_{187}$ N$_{e2}$. In myrosinase, departure of the aglycon without assistance from an enzymatic acid/base residue can be attributed either to substrate assistance via the sulfate group of the aglycon or to the good leaving group ability of the aglycon. According to our model based on the position of sulfate in the complex with gluco-hydroximolactam, a contribution of the sulfate group to catalysis is unlikely, because the oxygen atom next to the sulfur atom of the thioglycosidic linkage is that involved in the covalent bond to the nitrogen atom of glucosinolates. However, rotation of the sulfate group toward the thioglycosidic linkage during catalysis.

**FIG. 2. View of the active site with different inhibitors.** $F_o - F_c$ electron density maps contoured at 3 $\sigma$ based are based on a model of the native structure from which solvent molecules in the active site have been removed. Water molecules are shown as red spheres. The refined structures with the bound inhibitor are superposed, including water molecules, sulfate ions, inhibitor, and active site residues. The figure was generated with the program O (45). a, D-glucono-1,5-lactone. b, gluco-tetrazole. c, gluco-hydroximolactam. In presence of the inhibitor an additional sulfate ion is bound to the active site, and its hydrogen bonds are shown as dotted lines. d, 2-F-glucosyl enzyme covalently bound to Glu$_{409}$. The fluorine atom is shown in yellow. The pink arrow marks the water molecule that is believed to hydrolyze the glucosyl enzyme.

**FIG. 3. Conformation of different inhibitors bound to the active site of myrosinase.** The nucleophile is shown as well as Gln$_{187}$, which is at the position of the acid/base. The structure of the gluco-tetrazole complex is shown in red, the gluco-hydroximolactam inhibitor is in blue, together with the bound sulfate molecule. The gluconolactone is shown in green, and the covalently linked 2-F-glucose is in magenta. The drawing was made with MOLSCRIPT (40).
could allow a better positioning of the sulfate group in the vicinity of the glycosidic sulfur atom. Because of its low pK_a, the sulfate group is very unlikely to be protonated at neutral pH, which is optimal for the reaction (28, 41, 42). Nevertheless, intramolecular participation of the sulfate group cannot be fully excluded, because its pK_a could be shifted significantly in the enzyme active site. The observation that desulfo-glucocaparin, a substrate analogue that lacks the sulfate group, is neither an inhibitor nor a substrate (23) does not help to resolve the issue of the possible intramolecular participation of the sulfate group of glucosinolates; conceivably, this sulfate group is used by active site residues to induce the distorted conformation of the substrate required for catalysis. Myrosinase is inactivated by 2-F-GTL with a rate constant of 0.083 min^-1 and a K_i of 0.9 mM (5). The aglycon of 2-F-GTL is identical to that of glucotropaeolin, the natural substrate, evidencing that the aglycon does not need any additional “pull.” Again, this observation cannot be taken as evidence for or against a participation of the sulfate group, because optimal binding interactions of the enzyme with the aglycon can considerably increase the leaving group ability of the aglycon (43).

The enzyme-substrate complex must be very transient, because there is a difference of several orders of magnitude between the K_m of the myrosinase-catalyzed reaction and the K_i of the nonhydrolyzable substrate analogue C-GTL. On the one hand, it has not been possible to observe any electron density in the crystals of myrosinase for the slowly hydrolyzed substrate analogue 2-F-GTL where K_m for the inactivation is 0.9 mM (5), even at a soaking concentration of 100 mM. This suggests a K_i above 100 mM, considering this compound a competitive inhibitor. Because the glycosylation reaction can be carried out in the crystal this shows that the packing does not prevent catalysis. Furthermore, effects of the crystal packing on the active site are unlikely, because of the absence of crystal contacts in its vicinity and the rigidity of the active site reflected by low atomic temperature factors. On the other hand, hydrolysis of 2-F-GTL (k_i = 0.083 min^-1) is sufficiently slow that the enzyme-substrate complex can be observed in crystals that have been soaked with the inhibitor. The low K_m value for the inactivation in the presence of 2-F-GTL shows that substrate recognition is not abolished by the replacement of the 2-hydroxyl group by fluorine. Nevertheless, there is an increase in K_m of 1 order of magnitude compared with the natural substrate (K_m = 0.075 mM) (41). We have to conclude that in the case of myrosinase, the short lifetime of the Michaelis complex between myrosinase and glucosinolate is compensated by a high commitment toward hydrolysis.

The precise positioning of a water molecule above C-1 of the glucosyl enzyme intermediate by hydrogen bonding to Oe1 (d = 2.79 Å) and Ne2 (d = 3.27 Å) of Glu-187 promotes the last step of the reaction to a sufficient but not to an optimal extent (Fig. 7a). Binding of ascorbate slightly affects the position of this water molecule that now interacts with O-3 of ascorbate (d = 2.56 Å) and no longer with Glu-187 (d = 3.99 and 3.70 Å, Fig. 7b); it may thus be activated by (partial) deprotonation by ascorbate acting as a base. The pK_a of 4.1 of ascorbate at 25 °C suggests an optimum activation around that pH value, but we observed the same flat pH dependence of activity as described by Björkman and Lönnernald (28) or Palmieri et al. (41) in the presence or absence of 0.5 mM ascorbate using sinigrin as substrate (data comes from the cryoprotectant, b, ascorbate and gluco-hydroximolactam. The ascorbate competes with the sulfate ion that has both partial occupancies of 0.6 for the ascorbate and 0.4 for the sulfate, c, ascorbate bound to the 2-F-glucosyl enzyme. The water molecule that is activated by the ascorbate for an attack on the C-1 carbon of the glucose is indicated by a pink arrow.

**Fig. 4. The binding of ascorbate.** The structures have been obtained on crystals soaked with ascorbic acid and different inhibitors. Electron density maps as described for Fig. 2. Water molecules are shown as red spheres. The refined structures are shown, including ascorbate, water molecules, sulfate ions, glycerol, inhibitors, and active site residues. Hydrogen bonds involved in ascorbate recognition are shown as dotted lines. a, soak with ascorbate, the glycerol molecule
not shown). As described earlier for the sinigrin- \textit{S. alba} myrosinase system (41), the activation is about 2-fold. We assume that the pK$_a$ of the bound ascorbate is shifted toward neutral pH, so that the activation effect is not distinguishable from the general pH dependence of the activity.

So far there is no satisfactory explanation of the large reported differences in the degree of activation, ranging from 1.8- (28) to 400-fold (23) and depending on the source and isoform of myrosinase and on the substrate. The possible influence of other factors, such as the purification protocol or buffer system is as yet unclear. There might be minor differences in the active site of isoforms that could affect the relative affinities for substrate and activator and dramatically influence the fine balance between activation and inhibition, explaining the different degrees of activation. These differences do not show up in the few known sequences of myrosinases. The key residues of the active site involved in the interaction with ascorbic acid (Arg259 and the hydrophobic residues Phe371, Phe473, Tyr330, and Ile257) are strictly conserved.

We assume that the reaction catalyzed by myrosinase proceeds according to Fig. 8 (a and b) in the absence or presence of ascorbate. After formation of the enzyme-substrate (ES) complex governed by $k_1$ and $k_2$, the glucosyl enzyme (GE) is formed with rate constant $k_3$. The glucosyl enzyme is hydrolyzed into free glucose and free enzyme with rate constant $k_4$. The back reactions for the hydrolysis of the glucosyl enzyme and the cleavage of the substrate have been neglected. With these assumptions, Equations 1 and 2 describe the kinetic parameters of the reaction (38).

\begin{align}
K_m &= \frac{k_1 + k_2}{k_1 k_2 k_3 + k_1} \quad \text{(Eq. 1)} \\
V_{\text{max}} &= \frac{k_2 k_3}{k_2 + k_4} \quad \text{(Eq. 2)}
\end{align}

An action of ascorbate at the stage of substrate cleavage governed by $k_2$ can be excluded, since aglycon and ascorbate cannot bind at the same time in the active site. If $k_2$ was rate-limiting ($k_2 \ll k_3$), then $V_{\text{max}}$ and $K_m$ would remain constant if $k_3$ increases. If, however, deglycosylation is rate-limiting ($k_3 \ll k_2$), then an increase in $k_3$ in the presence of ascorbate should lead to an increase in $V_{\text{max}}$ and $K_m$, both proportional to $k_3$, as indeed observed: Michaelis-Menten constants for sinigrin of 23 and 250 $\mu$M have been measured for \textit{R. sativus} myrosinase in absence and presence of 0.5 mM ascorbate, respectively (29). In the same time, $V_{\text{max}}$ increased from 2.06 to 280 $\mu$mol/min/mg. A similar increase of $K_m$ in parallel to $V_{\text{max}}$ has been observed by Björkman and Lönnerdal (28). These results confirm a model where the deglycosylation step of the reaction catalyzed by myrosinase is rate-limiting and

![Fig. 5. Kinetics of reactivation in absence (v) or in presence of ascorbic acid (v, 0.1 mM; s, 1 mM). The plot shows recovered activity versus time.](image1)

![Fig. 6. A model of substrate binding. The model of the active site residues including the ball-and-stick model of bound sinigrin is shown (yellow). The positions of the gluco-hydroximolactam inhibitor and of the bound sulfate ion in the refined model on which the modeling is based are shown in cyan. The sinigrin molecule has been placed by hand and has been submitted to a crystallographic energy minimization using the gluco-hydroximolactam data set. The refinement constrained the sulfate and glucose part of the model to the experimental electron density while the geometry was regularized.](image2)
where this step is activated by ascorbate.

When a high concentration of ascorbate is used, there is binding competition between ascorbate and the substrate, resulting in a competitive inhibition, as observed by Shikita et al. (29). The additional hydrogen bond of the hydroxyl group at C-6 of the glucosyl group with the hydroxyl group at C-6 of ascorbate (Fig. 4, b and c) may increase the affinity of the binding site for ascorbate once the glucosyl enzyme has been formed. This is an advantage for the enzymatic function, considering the competition between binding of substrate and ascorbate.
This increase in affinity is schematized in Fig. 8b, where the rate constants \( k_{A1} \) and \( k_{A-1} \) for ascorbate binding to the glucosyl enzyme (GE) are not the same as the rate constants \( k_{A2} \) and \( k_{A-2} \) for ascorbate binding to the enzyme alone.

A scheme of the ascorbate-assisted cleavage of the thioglycosidic linkage by myrosinase is given in Fig. 8c. The glucosinolate binds to the active site, but the enzyme-substrate complex is very unstable and short-lived (I). The bound glucosinolate can be cleaved by the action of the nucleophile Glu409, without enzymatic activation of the thioglycosidic linkage (II). Upon formation of the glucosyl enzyme, a molecule of ascorbate binds to the active site and acts as a base, abstracting a proton from a water molecule (III) and thus enhancing its nucleophilic attack at the anomeric center (IV). Finally, glucose and ascorbate are released from the active site.

In typical retaining glycosidases, the nucleophile and general acid/base residues are separated by 4.8 to 5.3 Å (6). In myrosinase, Gln187 is located precisely at the same position (5.0 Å from the nucleophile oxygen) as the acid/base residue of related O-glycosidases. In contrast, ascorbate is placed with its O-3 oxygen 7.0 Å away from Glu409. This larger distance is easily explained by the alleviated distance constraints for ascorbate, as compared with the acid/base residue of O-glycosidases, which must be at a position that also allows activation of the leaving group by proton transfer to the glycosidic oxygen.

To the best of our knowledge, myrosinase is the first example of an enzyme catalyzing a hydrolysis where an external cofactor is recruited in the middle of a reaction path to ensure base catalysis. Furthermore, activation of myrosinase appears to also be the first case where ascorbic acid is not used as reducing agent. The effect of ascorbate in contrast to 2-dehydroascorbate (26) could be the basis of a regulation mechanism for myrosinase activity as a function of the redox potential in the cell.

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