Role of methionine in the active site of α -galactosidase from *Trichoderma reesei*

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α -Galactosidase from *Trichoderma reesei* when treated with H_2O_2 shows a 12-fold increase in activity towards *p*-nitrophenyl α -D-galactopyranoside. A similar effect is produced by the treatment of α -galactosidase with other non-specific oxidants: $NaIO_4$, $KMnO_4$ and $K_2S_2O_8$. In addition to the increase in activity, the Michaelis constant rises from 0.2 to 1.4 mM, the temperature coefficient decreases by a factor of 1.5 and the pH-activity curve falls off sharply with increasing pH. Galactose (a competitive inhibitor of α -galactosidase; K_i 0.09 mM for the native enzyme at pH 4.4) effectively inhibits oxidative activation of the enzyme, because the observed activity changes are related to oxidation of the catalytically important methionine in the active site. NMR measurements and amino acid analysis show that oxidation to methionine sulfoxide of one of five methio-

nines is sufficient to activate α -galactosidase. Binding of galactose prevents this. Oxidative activation does not lead to conversion of other H_2O_2 -sensitive amino acid residues, such as histidine, tyrosine, tryptophan and cysteine. The catalytically important cysteine thiol group is quantitatively titrated after protein oxidative activation. Further oxidation of methionines (up to four of five residues) can be achieved by increasing the oxidation time and/or by prior denaturation of the protein. Obviously, a methionine located in the active site of α -galactosidase is more accessible. The oxidative-activation phenomenon can be explained by a conformational change in the active site as a result of conversion of non-polar methionine into polar methionine sulfoxide.

INTRODUCTION

α -Galactosidase (melibiase EC 3.2.1.22) hydrolytically cleaves terminal α -D-galactopyranosyl residues from oligosaccharides, branched polysaccharides, such as galactomannans and galactoglucomannans, and carbohydrate moieties of glycoproteins. α -Galactosidases have been purified to homogeneity from various sources: higher plants [1,2], yeasts [3], fungi (*Aspergillus awamori* X100/D27 [4], *Morella vinacea* [5] and *Aspergillus niger* [6]) and *Escherichia coli* [7]. For several α -galactosidases, such as those obtained from *Saccharomyces carlsbergensis* and *Streptococcus mutans*, amino acid sequences have previously been determined [8,9]. However, the structures of active sites and catalytically important amino acid residues will largely remain unknown.

Some α -galactosidases have been found to carry a functionally important thiol group. In the present work on α -galactosidase from *Trichoderma reesei*, we identified a methionine residue near its active site. Oxidation of this methionine altered the kinetic properties of the enzyme and enhanced its activity towards the substrate *p*-nitrophenyl α -D-galactopyranoside (PNPG).

Methionines have previously been discovered in the active sites of some carbohydrases, namely peptides isolated from the *Streptomyces* T7 β -xylosidase [10], β -glucosidase from *Aspergillus wentii* [11], α -glucosidase from rabbit small intestine [12] and β -galactosidase from *E. coli*. Irreversible inactivation of the latter by β -D-galactopyranosyl methyl-*p*-nitrophenyltriazine revealed the presence of Met-500 in the active site [13]. However, enhanced activity as the result of oxidation of methionine in the active site has not thus far been reported.

MATERIALS AND METHODS

PNPG, *p*-chloromercuribenzoate (PCMB), *p*-hydroxymercuribenzenesulphonic acid (PHMBS), catalase, horseradish peroxidase (HRP), arabinose, glucose, NaCN, α -mannosidase from jack beans, BSA and $NaBH_4$ were purchased from Sigma. Galactose and melibiose were from Koch Light. Ethyl ethanethiosulphonate (TSF) and Ellman reagent were kindly provided by Dr. E. Kropachev and Dr. N. Soroka, St. Petersburg Nuclear Physics Institute. *p*-Nitrophenyl α -thiogalactopyranoside (PNPTG) was synthesized as described by Voznyi et al. [14]. All other reagents were of analytical grade.

Purification of α -galactosidase

The extracellular protein was isolated from a culture of the mesophilic fungus *T. reesei* and purified according to the following procedure: after 72 h of cultivation, the culture medium was centrifuged and the supernatant concentrated 30-fold by ultrafiltration on hollow fibres. The filtrate was then desalted on a Sephadex G-50 column equilibrated in 10 mM sodium phosphate buffer, pH 6.7, and loaded on a DEAE-TOYOPEARL column pre-equilibrated in the same buffer. Elution was achieved with a linear gradient (10 mM–0.5 M) of sodium phosphate buffer, pH 6.7. Fractions containing α -galactosidase activity were pooled, concentrated by ultrafiltration on an Amicon PM30 membrane and transferred to 20 mM sodium acetate buffer, pH 4.1, by gel filtration on a Sephadex G-50 column. At the final stage of purification, the enzyme solution was applied to a TSK SP 5 PW column (Pharmacia-LKB) equilibrated in 20 mM

Abbreviations used: HRP, horseradish peroxidase; PCMB, *p*-chloromercuribenzoate; PHMBS, *p*-hydroxymercuribenzenesulphonic acid; PNPG, *p*-nitrophenyl α -D-galactopyranoside; PNPTG, *p*-nitrophenyl α -thiogalactopyranoside; TSF, ethyl ethanethiosulphonate; ESI-MS, electrospray ionization mass spectrometry; DSS, 4,4-dimethyl-4-silapentanesulphonate.

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sodium acetate buffer, pH 4.1, and eluted with a linear gradient (0–0.5 M) of NaCl in the same buffer. Fractions containing α -galactosidase were pooled, desalted and concentrated on an Amicon PM30 membrane. All purification steps were performed at 4 °C [15].

SDS/PAGE analysis showed that the protein had a molecular mass of 54 kDa. The same value was obtained by analytical gel filtration on a TSK G-3000 SW column (Pharmacia–LKB). The isoelectric point determined using Servalyt Precot plates was 5.25. These analyses showed that the enzyme was homogeneous and monomeric; reversed-phase chromatography (Lichrosorb C-18 Pharmacia–LKB column, starting buffer pure water, eluent acetonitril) confirmed this.

The same analysis of oxidatively activated enzyme revealed that its molecular mass, isoelectric point and monomeric construction remained unchanged during activation.

The molecular mass of partly deglycosylated α -galactosidase was determined by electrospray ionization mass spectrometry (ESI–MS). For this, α -galactosidase was subjected to additional purification on a Mono S HR5/5 Pharmacia–LKB column, with 20 mM sodium acetate, pH 4.1, as starting buffer, and the same sodium acetate concentration plus 0.5 M NaCl as eluting buffer. Elution was performed with a linear gradient (0–0.5 M) of NaCl. The protein was then desalted in water on a Sephadex G-25 Fine column, dialysed against deionized water for 3 days and finally lyophilized.

Protein determination

Protein was measured by the Lowry procedure [16], with BSA as standard. In addition, the concentration of pure α -galactosidase was determined spectrophotometrically, using a specific absorption coefficient at 278 nm of $1.86 \text{ litre} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$.

Enzyme assay

α -Galactosidase activity was evaluated using PNPG as substrate [17]. One unit of enzyme activity is defined as the amount of enzyme that hydrolysed $1 \mu\text{mol}$ of substrate/min at 37 °C. For native enzyme, the assay with PNPG was carried out at a substrate concentration of 3 mM. For oxidatively activated enzyme, the concentration of PNPG was increased tenfold (i.e. up to 30 mM). These conditions produced saturation of both forms of the enzyme at any pH above 3. In the case of partially activated enzyme samples (experiments on time-dependence of oxidative activation), activity was determined as a corresponding Lineweaver–Burk extrapolation, using three experiments at 0.75, 1.5 and 3 mM concentrations of PNPG.

The activity of native α -galactosidase on melibiose was determined by measuring released glucose by the glucose oxidase method [18]. Activity was determined as a Lineweaver–Burk extrapolation, using 1–10 mM melibiose.

If α -galactosidase samples contained mercurial compounds (HgCl_2 , PHMBS, PCMB), the reaction mixture was supplemented with 50 mM cysteamine. In controls, this concentration did not affect the enzyme activity (incubation time less than 15 min).

Enzyme activity data are presented in three ways: (i) percentage of initial activity, in experiments on enzyme inhibition and enzyme stability (Figures 2 and 7); (ii) relative activity, in experiments on oxidative activation (ratio of activity after and before the activation) (Figures 1, 8 and 10); (iii) specific activity, i.e. activity of 1 mg of protein, expressed in units/mg of protein (Figure 3 and Table 1).

Amino acid analysis

Acid hydrolysis of α -galactosidase was performed by incubating 1.25 mg/ml protein with 5.7 M HCl and 10 mM phenol at 110 °C for different periods of time. Tryptophan was determined spectrophotometrically as described by Edelhoch [16]. Alkaline hydrolysis was carried out by incubating 10–11 mg/ml protein in 4 M NaOH at 110 °C for different periods of time. Amino acid composition was determined with a Biotronik amino acid analyser.

α -Mannosidase treatment

α -Galactosidase (1 mg/ml of 50 mM sodium acetate buffer, pH 4.5) was mixed with 2 μg of α -mannosidase and incubated for 12 h at 37 °C.

Carbohydrate determination

Composition analysis of neutral sugars was performed by GLC/MS, with arabinose as standard. The protein was hydrolysed in 2 M HCl at 100 °C for 3 h, and the released sugars were characterized as alditol acetates [19]. A DB-5 column with a temperature gradient of 180–250 °C and an Incos-50 mass spectrometer (Finnigan MAT) were used. Galactosamines were determined with an amino acid analyser after 16 h of hydrolysis of the protein in 4 M HCl at 100 °C [20].

ESI-MS determination of molecular mass

Purified α -galactosidase was analysed as previously described [21], on a VG BioTech BioQ mass spectrometer (VG BioTech Altrincham, Cheshire, U.K.) on the basis of ESI and using a quadrupolar mass analyser with a maximum mass-to-charge value, m/z , of 4000.

Protein denaturation

For denaturation by guanidine, 10 mg/ml α -galactosidase was incubated in buffer containing 6 M guanidium chloride, 2 mM EDTA and 0.02 M Pipes, pH 6.4, for 30 min. The protein was dialysed against water and lyophilized.

Acid denaturation was carried out by dissolving α -galactosidase in water to a concentration of 10 mg/ml, acidifying the solution with 1 M HCl to pH 0.7 followed by incubation at 40 °C for 1 h. Thereafter the protein was lyophilized and stored for use in subsequent experiments.

Oxidation-induced activation of α -galactosidase

α -Galactosidase was incubated in 50 mM potassium phosphate buffer, pH 6.4, containing 0.5 M H_2O_2 , at 20 °C. Unless otherwise stated, these were the standard conditions for all activation experiments. To protect the -SH group from oxidation, a 20-fold molar excess of PHMBS over α -galactosidase was added. Incubations were continued until the activity of the enzyme stabilized (approx. 1.5–2 h under standard conditions). To prepare activated enzyme, H_2O_2 was destroyed by catalase as described below. Cysteamine (10 mM) was then added to the solution to remove mercurial compounds. Protein was dialysed against two changes of 10 mM sodium acetate buffer, pH 5.5, for 24 h, after dialysis against 1 vol. of distilled water, and lyophilized.

L-Methionine was oxidized under standard conditions and its

conversion into methionine sulfoxide was monitored by ^1H -NMR spectroscopy and amino acid analysis.

Removal and assay of H_2O_2

Destruction of excess H_2O_2 after incubation was achieved by one of the following procedures. To avoid excess dilution of sample before analysis, the reaction mixture was supplemented successively with 1% (v/v) 10 mM catalase in 50 mM potassium phosphate buffer, pH 7, 1% (v/v) butan-2-ol (antifoam), and again with 1% catalase, at 30 s intervals with thorough mixing. When sample dilution was acceptable (i.e. before enzyme assay), an aliquot no greater than 2% (v/v) final concentration was added directly to 0.2 μM catalase solution in potassium phosphate buffer, pH 7. H_2O_2 content was determined by a colorimetric peroxidase reaction. The test sample was mixed with an equal volume of a solution containing 1 mM aniline, 1 mM amidopyrene and 10 μM HRP in 0.2 M sodium acetate buffer at pH 5.0. The aniline/amidopyrene mixture was oxidized to yield a purple colour which was monitored spectrophotometrically at 530 nm [22]. The catalase-treated solutions contained less than 1 μM H_2O_2 , no matter which treatment was used.

Visible absorption and fluorescence spectroscopy

Visible absorption spectra were obtained with an M40 Specord spectrophotometer (Carl Zeiss), and fluorescence spectra with an F-4010 Hitachi spectrofluorimeter.

^1H -NMR spectroscopy

NMR spectra were recorded with a HX-270 Bruker Spectrospin spectrometer operating at 270 MHz with a proton probe. The protein samples for NMR measurements contained 20–25 mg of α -galactosidase, 20 mM potassium phosphate buffer (pH 6.4) and 50 μM 4,4-dimethyl-4-silapentanesulphonate (DSS) used as internal standard, in 0.5 ml of 99.8% $^2\text{H}_2\text{O}$. Other components, such as PHMBS or sugars, were added as portions of corresponding concentrated solutions. The $^1\text{H}^2\text{HO}$ line was pre-saturated for 0.5 s with a decoupler operating at 24 dB in CW mode. Data were acquired after 75° pulls into 16 K points, with a spectral width of 10 kHz and 2053 scans including the first five dummy scans. The spectra were Lorentz-broadened by 1 Hz. DSS signal was used for adjustment of phase and amplitude parameters in order to obtain correct differential spectra.

NMR spectra of free L-methionine were recorded from samples containing 10 mM L-methionine in $^2\text{H}_2\text{O}$, pH 6.2–6.4, as judged by use of a pH indicator strip. DSS (1 mM) was used as internal standard. $^1\text{H}^2\text{HO}$ presaturation time was 5 s, the total scan number was 64, there were no dummy scans and other settings were the same as above. Oxidation of methionine in α -galactosidase was determined using ^1H NMR by comparing the integrated differential α -galactosidase spectra within 3.5–1.5 p.p.m. and integrated ordinary α -galactosidase spectra within 4.5–0.0 p.p.m. The number of observable protons in α -galactosidase was calculated using the Table of Chemical Shifts [23] and the data on amino acid content.

RESULTS

Oxidation-induced activation of α -galactosidase

Activation of α -galactosidase by oxidation occurs via changes in some of its kinetic properties caused by the action of bi- and multi-electron oxidants: H_2O_2 , KMnO_4 , $\text{K}_4\text{S}_2\text{O}_8$, NaIO_4 . A most interesting effect is an increase in the enzyme activity towards the

Figure 1 Oxidation-induced activation of α -galactosidase with various oxidants

α -Galactosidase was present at a concentration of 10 μM in the incubation cell. $\circ$, Activation by H_2O_2 under standard conditions (see the Materials and methods section), with no mercury agents or protecting inhibitors added; $\square$, activation by H_2O_2 in the presence of 0.2 mM PHMBS to protect the active -SH group; $\triangle$, activation by H_2O_2 in the presence of 15 μM HgCl_2 instead of PHMBS; $\bullet$, activation by H_2O_2 in the presence of 0.2 mM PHMBS and 20 mM D-glucose; $\blacktriangle$, activation by H_2O_2 in the presence of 0.2 mM PHMBS and 20 mM D-glucose; $\times$, activation by 0.1 mM KMnO_4 in 50 mM sodium acetate buffer, pH 4.8; $\diamond$, activation by 0.2 M $\text{K}_2\text{S}_2\text{O}_8$ in 50 mM sodium acetate, pH 4.8; $\blacklozenge$, activation by 0.01 M NaIO_4 in Tris/HCl buffer, pH 7.9. Inset: pH-dependence of α -galactosidase activity after 15 min incubation with 0.5 M H_2O_2 at 18 °C.

PNPG substrate. Results from activation experiments summarized in Figure 1 show that the relative enhancement of the activating effect of the above oxidants follows the order: $\text{NaIO}_4 < \text{K}_4\text{S}_2\text{O}_8 < \text{H}_2\text{O}_2 < \text{KMnO}_4$. The H_2O_2 -activation effect is increased under weakly acidic conditions (below pH 5) (see the inset of Figure 1).

Long-term exposure of the enzyme to oxidizing conditions leads to irreversible loss of activity, the onset of irreversible inactivation being inversely proportional to oxidant activity [Figure 1, results for H_2O_2 (no additions), KMnO_4 and $\text{K}_4\text{S}_2\text{O}_8$]. Therefore in subsequent activation studies, we selected H_2O_2 as the oxidant because it can be readily removed from the enzyme solution by gel filtration on a Sephadex G-25 column or destroyed by catalase. After removal or destruction of H_2O_2 , the activated enzyme retains its newly acquired properties.

In addition, we found that the presence of 0.1–0.8 M H_2O_2 did not affect the rate of PNPG hydrolysis during the time required for enzyme assay (this time was much shorter than the enzyme activation period; results not shown). Overall, these observations suggest that peroxide achieves its effect by changing certain properties of the enzyme rather than by directly affecting the rate of substrate hydrolysis.

The activated α -galactosidase was almost as stable as the native enzyme (Figure 2). At the optimal pH (6.0–6.1), solutions of both native and activated enzyme retained their activities for several days when stored at room temperature. After lyophilization and subsequent dissolution, activities of the native and activated enzymes were 80–85% and 75–80% of original respectively.

Changes in properties of α -galactosidase after oxidation-induced activation

After treatment with H_2O_2 , the activity of α -galactosidase towards PNPG was 12-fold higher at 37 °C between pH 4 and 5 (Figure 1, inset). The pH- and temperature-dependence of the

Figure 2 pH-dependent stability of α -galactosidase at 20 °C

(a) Native enzyme; (b) oxidatively activated enzyme.

Figure 3 pH-dependence of α -galactosidase activity at 18 °C

○, Native enzyme; □, H_2O_2 -treated enzyme in the presence of 0.2 mM PHMBS under conditions described in Figure 1; △, enzyme treated with H_2O_2 in the presence of 0.2 mM PHMBS and 20 mM galactose; ●, activation percentage calculated for the above sample for the whole pH region.

catalytic activity was also affected. Activity fell off more sharply with increased pH than observed in the untreated enzyme (Figure 3). The mean temperature coefficient for PNPG hydrolysis within the range 17–37 °C fell from 2.5 to 1.7 per 10 °C decrement (results not shown), i.e. the energy barrier in the enzyme–substrate complex was lowered. The Michaelis–Menten constant K_m and the dissociation constant K_i for complexes of enzymes with competitive inhibitors increased approximately 10-fold (Table 1). This indicates that the activated α -galactosidase acquires a somewhat lower affinity for galactose-containing substrates and inhibitors.

Table 1 Activity, K_m and K_i for native and oxidatively activated α -galactosidase

The enzyme was in 50 mM acetate buffer, pH 4.4, at 37 °C. Activity is expressed in units/mg of protein; variation was 10%. K_m and K_i are expressed in mM; variation was 15%.

Substrate or inhibitor	Native enzyme			Activated enzyme		
	Activity	K_m	K_i	Activity	K_m	K_i
PNPG	47	0.20	—	588	1.4	—
Galactose	—	—	0.091	—	—	2.2
PNPTG	—	—	0.11	—	—	2.9
Melibiose	6.2	3.3	3.1*	—†	> 50	48*

* Melibiose was used as competitive inhibitor to PNPG.

† The value was not determined.

Figure 4 ESI-MS spectrum of partially deglycosylated α -galactosidase

Analysis of Michaelis–Menten kinetics showed that D-galactose was a competitive inhibitor of α -galactosidase (results not shown). The K_i was pH-dependent, falling from 100 μM at pH 4.0 to 20 μM at pH 7.0. There was a similar decrease in K_m for PNPG from 200 to 35 μM .

As shown in Figure 1, 20 mM galactose (pH 6.2) effectively prevented oxidation-induced activation. In contrast, glucose at the same concentration and pH was without effect, even at concentrations up to 0.1 M. This supported the hypothesis that the blocking of oxidative activation by D-galactose was due to competitive inhibition.

Table 2 Amino acid compositions of α -galactosidase after acid and alkaline hydrolysis

The average precision was 3%; the values are rounded off to integers. Predenatured enzyme was denatured with either 7 M guanidinium chloride (GdmCl) or 0.2 M HCl at 40 °C and then treated for 1.5 h with H_2O_2 under standard conditions. Detailed experimental conditions are given in the Materials and methods section. Tryptophan was analysed spectrophotometrically [16]. NA, not analysed; Met-SO, methionine sulfoxide; Met-SO₂, methionine sulphone.

Amino acid	Composition (mol/mol of enzyme)					
	Native enzyme				Predenatured enzyme after alkaline hydrolysis	
	Acid hydrolysis		Alkaline hydrolysis		GdmCl	HCl
	24 h	48 h	16 h	38 h		
Asx	62	62	62	61	61	62
Thr	29	29	6	2	4	3
Ser	30	29	10	6	10	10
Glx	27	27	27	27	27	27
Pro	25	24	21	21	21	22
Gly	43	43	50	54	50	51
Ala	46	46	60	61	62	62
Cys	7	7	NA	NA	NA	NA
Val	21	23	12	15	13	13
Ile	15	16	8	8	8	8
Leu	36	36	36	37	36	36
Tyr	16	16	15	16	15	14
Phe	11	12	12	12	12	12
Lys	12	12	13	12	14	13
His	10	10	10	10	9	10
Arg	15	15	15	16	15	16
Trp	19	19	NA	NA	NA	NA
Met	5	5	5	5	2	1
Met-SO	—	—	—	—	3	4
Met-SO ₂	—	—	—	—	—	—

We used galactose protection to prove that the observed changes in α -galactosidase properties after oxidation occurred in parallel with the enhancement in activity. The pH-dependence of enzyme activity (Figure 3) serves as an example of this. The percentage of activated enzyme protected by 20 mM galactose, calculated from pH-activity measurements, was constant throughout the whole pH range. This indicates that the changes in pH-activity could not occur independently of oxidative activation. A similar relationship was noted for the temperature-dependence coefficient and K_m (results not shown).

Effect of the H_2O_2 on the amino acid composition of α -galactosidase

In order to determine oxidation-induced changes in the amino acid composition of α -galactosidase, total amino acid contents of native and activated enzymes were compared. As we predicted minimal composition changes, precise values for the molecular mass and percentage carbohydrate content of the protein were calculated before comparative studies to ensure reliable assessment.

The molecular mass of α -galactosidase was determined by ESI-MS. Spectra of the native α -galactosidase were difficult to interpret because of multiplets characteristic of glycoproteins with carbohydrate microheterogeneity [24]. Therefore the enzyme was partially pre-deglycosylated by treatment with α -mannosidase, permitting us to obtain reasonable spectra (Figure 4) and thereby determine an average molecular mass of $51\,207 \pm 3$ Da.

The residual sugar composition of the partially deglycosylated enzyme was then evaluated as described in the Materials and methods section, and the monosaccharide ratio

Table 3 Precise data on conversions of oxidizable amino acids during 1.5 h of incubation of α -galactosidase with H_2O_2 under standard conditions

Detailed experimental conditions are given in the Materials and methods section. Amino acids were analysed after alkaline hydrolysis, except for cysteine which was analysed independently after acid hydrolysis. An average precision of 3% was obtained. A, native α -galactosidase; B, α -galactosidase treated with H_2O_2 without predenaturation, in the presence of 20 mM glucose; C, α -galactosidase treated with H_2O_2 with no predenaturation, in the presence of 20 mM galactose; D, α -galactosidase treated with H_2O_2 after predenaturation with 0.2 M HCl at 40 °C; E, α -galactosidase treated with H_2O_2 after predenaturation with 7 M guanidinium chloride. Met-SO, methionine sulfoxide. * All values in the row are significantly different ($P < 0.001$) (Student's *t* test).

Amino acid	Composition (mol/mol of enzyme)				
	A	B	C	D	E
Tyr	15.32	14.94	15.58	15.12	14.40
Cys	6.93	6.88	7.12	—	—
His	9.90	10.25	10.05	9.75	10.18
Met*	5.06	3.94	4.31	1.89	0.95
Met-SO*	0.0	0.95	0.62	2.94	4.22

(Man/Gal/Glc/NAcGlc) estimated to be 10:1:0.5:1 (with an overall carbohydrate content of 8%, w/w).

The amino acid compositions of α -galactosidase obtained after acid and alkaline hydrolysis are shown in Table 2. Under mild conditions, several amino acids may be potential targets for oxidants (in order of oxidation sensitivity): methionine, cysteine, tryptophan, cystine, histidine and tyrosine [19]. We carried out a thorough analysis of the above residues in α -galactosidase after oxidation. Table 3 shows that of the oxidizable amino acids,

Figure 5 Determination of oxidized methionine in α -galactoside by means of differential ^1H -NMR spectroscopy

1, Difference spectrum between the spectra of native L-methionine and its primary oxidation product, methionine sulfoxide, obtained after 1 h of incubation in the presence of 0.1 M H_2O_2 at pH 6.5; 2, the same spectrum, Lorentz-broadened by 14 Hz; 3, difference spectrum between spectra of the native and oxidized α -galactosidases. The enzyme was oxidized with 0.1 M H_2O_2 for 3 days in the presence of 0.2 mM PHMBS as described in the Materials and methods section.

only the methionine conversion is statistically significant. It is known that methionine is oxidized to methionine sulfoxide, and with time or under stronger conditions, to methionine sulphone [19,25].

In this study, methionine sulfoxide was detected by differential ^1H -NMR spectroscopy after prolonged oxidative activation of α -galactosidase (Figure 5), a finding confirmed by amino acid analysis. Both these techniques were used together in kinetic studies of the oxidative activation of α -galactosidase (see Figure 8).

The appropriate choice of protein hydrolysis method was critical. Whereas acid hydrolysis is satisfactory for amino acid determination in the native enzyme, it is inappropriate for analysis of methionine oxidation products. Acid hydrolysis was carried out with the addition of 0.01% 2-mercaptoethanol to prevent methionine decomposition [25]. However, 2-mercaptoethanol is capable of reducing methionine sulfoxide to methionine [26]. Methionine sulfoxide and methionine sulphone can also undergo other transformations on acid hydrolysis [26]. For these reasons we used alkaline hydrolysis. As shown in Table 2, methionine was quantitatively converted into methionine sulfoxide by the action of H_2O_2 on the denatured enzyme. Therefore a 16 h alkaline hydrolysis was used to determine methionine and

Figure 6 PCMB titration of α -galactosidase

Enzyme concentration was 0.1 mM and PCMB 1 mM; titration was carried out in 50 mM potassium phosphate buffer, pH 6.6. (a) Visible absorbance spectrum of α -galactosidase (1) and differential spectra of its conjugate with PCMB against native enzyme (2–4) obtained at 5 min intervals. (b) Time-dependent titration of α -galactosidase with PCMB, as monitored by A_{252} : 1, Native enzyme; 2, native enzyme + 10 mM galactose; 3, oxidatively activated enzyme; 4, oxidatively activated enzyme + 10 mM galactose.

methionine sulfoxide in experiments on α -galactosidase oxidation.

A low recovery of serine and threonine and concomitant increase in alanine and glycine are observed when alkaline hydrolysis is used because the prominent portion of serine and threonine are *O*-glycosylated. Such residues are typically converted under alkaline conditions into alanine and β -aminobutyric acid and partially into glycine [4].

Table 3 shows that incubation of native α -galactosidase (1.5 h in 50 mM potassium phosphate, pH 6.2, in the presence of 2 mM PHMBS) with 0.5 M H_2O_2 leads to the oxidation of approx. 1 mol of methionine to methionine sulfoxide per mol of protein (column B). Galactose (20 mM) exerts a protective effect, given the 3% variation of these measurements (column C). Under the conditions used, oxidizable aromatic residues (other than tryptophan, which is considered separately below) would not be oxidized even if the enzyme was denatured. The constant composition of cysteine indicates that disulphide bonds were also unaffected.

Behaviour of the active -SH group during oxidation-induced activation of α -galactosidase

Quantitative determination of the amino acid composition of the activated and native enzymes appeared to be poorly applicable to the estimation of minor changes in the number of thiol groups. Thiol groups are sensitive to different oxidants, including H_2O_2

Figure 7 Inactivation of α -galactosidase with TSF and subsequent partial re-activation with excess cysteamine

α -Galactosidase (10 μ M) was incubated in 50 mM phosphate buffer at 18 °C. Arrows indicate the moment of reagent addition: 5 mM TSF and 50 mM cysteamine. (a) Native enzyme; (b) oxidatively activated enzyme.

[19,27]. In our first report on oxidation-induced activation of α -galactosidase [28], we proposed that a catalytically significant -SH group was a major target for modification. This led us to examine further the behaviour of the thiol group during protein activation.

Quantitative titration of the enzyme with PCMB gave similar results for the native and activated enzymes: approximately one active -SH group per molecule was found (Figure 6). The -SH group concerned probably belongs to the active site and is essential for catalysis. Quantitative titration of this group with mercurial compounds causes almost complete inactivation of the enzyme, and galactose effectively reduces the rate of this inactivation (results not shown).

Inactivation of α -galactosidase with TSF and subsequent re-activation with excess cysteamine further supports our conclusions that oxidative activation does not affect the -SH group (Figure 7). Indeed, TSF reacts with the active -SH group to form an inactive disulphide [29]. The added excess thiol is involved in the sulphide-disulphide exchange [29,30], leading to a 50% recovery of the active thiol group. These effects were the same for the native and oxidatively activated α -galactosidases.

Perhaps the best proof of our hypothesis that oxidation-induced activation is not associated with modification of an -SH group is the observation that α -galactosidase is activated by H_2O_2 in the presence of PHMBS or PCMB which bind the thiol group (Figure 1). As shown by these experiments, activation does not decrease but instead reaches a maximum, whereas inactivation is almost completely blocked. It should be noted that PCMB titration of α -galactosidase that had been completely inactivated by prolonged H_2O_2 treatment did not reveal a free -SH group. Therefore it may be concluded that oxidative activation does not affect the thiol group. Conversely, oxidation of the active -SH group may be proposed as being the cause of the inactivation caused by prolonged incubation with H_2O_2 .

Protective effect of galactose on methionine residues in H_2O_2 treatment of α -galactosidase

We report above that galactose essentially inhibits oxidation-induced activation of α -galactosidase. Therefore several studies were conducted to establish a direct relationship between inhibited activation and protection against oxidation of methionine residues. Table 3 shows that addition of 20 mM galactose to the incubation mixture reduces conversion of methionine into methionine sulfoxide (over 1.5 h) from about 1 to 0.6 residue/mol of protein.

Figure 8 Comparison of changes in α -galactosidase activity and methionine-methionine sulfoxide conversion on oxidation-induced activation, as monitored by differential 1H NMR and amino acid analysis

Samples were incubated with 50 mM H_2O_2 in 99.8% 2H_2O /20 mM potassium phosphate buffer, pH 6.4, containing 1 mM PHMBS and 20 mM sugar [α -glucose (open symbols) or α -galactose (closed symbols)]. $\circ$, $\bullet$, Time-dependent activity; $\triangle$, $\blacktriangle$, time-dependent methionine-methionine sulfoxide conversion, as determined by NMR. Enlarged symbols, at about 100 h represent data from an independent test on activity and conversion; the sample contained no glucose. $\square$, $\blacksquare$, Methionine sulfoxide determined by amino acid analysis; $\diamond$, $\blacklozenge$, methionine determined by amino acid analysis. The x-axis is a shifted log scale.

Galactose-inhibited conversion of methionine during α -galactosidase activation was examined in greater detail by kinetic studies, using a combination of differential 1H NMR and amino acid analysis. Two samples of 20 mg of α -galactosidase each (0.7 ml of 2H_2O , 20 mM potassium phosphate buffer, pH 6.4, 1 mM PHMBS and 50 mM H_2O_2) were incubated at 18 °C in the presence of either 20 mM galactose or 20 mM glucose. Each sample was divided into two unequal portions of 0.5 and 0.2 ml. The larger portion was placed into an NMR tube and the smaller into a plastic microtube. Sample aliquots of 15 μ g and 1 mg were pooled from the NMR tube and microtubes for activity measurement and amino acid analysis respectively at defined time intervals. H_2O_2 was destroyed by catalase as described in the Materials and methods section. Immediately after each sampling, the 1H -NMR spectrum of the larger portion was measured. The number of methionine sulfoxide and methionine residues was determined by differential NMR and amino acid analysis after alkaline hydrolysis, as outlined above (Figure 8). It was concluded that one modified methionine residue is needed for the formation of an activated enzyme molecule.

Search for traces of oxidation-induced modification of aromatic amino acid residues

Tryptophan, tyrosine and histidine have been proposed as possible targets for oxidants, such as H_2O_2 [19], although they are less sensitive than methionine or cysteine. To prove that these amino acids remain unchanged during oxidative activation of α -

Figure 9 Comparison of spectra of aromatic regions of native and activated α -galactosidases

(a) Visible absorbance spectra of (1) native enzyme and (2) oxidatively activated enzyme. The spectra are artificially shifted along the y -axis. (b) Fluorescence spectra: 1 and 2 are the same as in (a). — Fluorescence excitation at 275 nm; ----, excitation at 290 nm; the spectra are artificially shifted along the y -axis. (c) ^1H -NMR spectra: 1 and 2 are the same as in (a). Solid arrows indicate points of minor difference at 6.8 and 7.0 p.p.m. 3, Difference between spectra 1 and 2; dashed arrows indicate the direction of spectral shifts.

galactosidase, we compared absorption, fluorescence and NMR spectra of the native and activated enzyme. Examination of the visible spectral regions characteristic of aromatic amino acids revealed no differences between the native and oxidized proteins (Figures 9a and 9b), indicating that tryptophan and tyrosine residues were unaffected. A thorough comparison of the ^1H -NMR aromatic region (6.5–9 p.p.m.) revealed a weak downfield shift of probably one tyrosine residue (Figure 9c). However, this shift is difficult to assign to chemical changes; rather it may be due to a conformation rearrangement that takes place during oxidative activation of the enzyme. Amino acid analysis also confirmed lack of effect of oxidation on aromatic acids (Table 3).

Examination of the effect of HRP on oxidative activation

Tressel and Kosman [31] established that plant peroxidase caused activation of glucose oxidase as a result of dityrosine intra-globular link formation, leading to stabilization of the enzyme active conformation. In spite of the fact that the corresponding

Figure 10 Effect of HRP on oxidative activation of α -galactosidase

(a) Normal oxidative activation compared with that in the presence of HRP and cyanide. ●, 0.5 mg/ml native α -galactosidase in 50 mM, acetate buffer, pH 5.4, containing 0.1 mM PCMB (for -SH group protection) and 0.5 M H_2O_2 at 18 °C; ■, as above plus 50 units/ml HRP; ▲, plus 1 mM NaCN. (b) SDS/PAGE of the native and oxidatively activated α -galactosidase. Lane 1, α -galactosidase, oxidatively activated under the conditions described in (a); lane 2, α -galactosidase, oxidatively activated under standard conditions (see the Materials and methods section); lane 3, native α -galactosidase; lane 4, protein standards: phosphorylase a (98 kDa), BSA (67 kDa), ovalbumin (45 kDa), α -chymotrypsin (25 kDa).

control confirmed that our preparation of α -galactosidase contained practically no traces of HRP (less than 0.005 pyrogallol units per mg of dry protein; results not shown), we investigated the possible effect of HRP on oxidative activation of α -galactosidase. Three samples were compared: the first contained native α -galactosidase, the second contained, in addition, a large amount (50 units/ml) of HRP and the third contained native α -galactosidase and 1 mM NaCN, a known inhibitor of peroxidases [32].

The time-dependence of α -galactosidase activity in this experiment (Figure 10a) showed that neither high concentrations of HRP nor cyanide really influenced oxidative activation of α -galactosidase.

Sample 1 from above was incubated up to maximum activation level (Figure 10a, closed circles). Samples of activated and native enzyme were then also examined by SDS/PAGE. Figure 10(b)

shows that oxidative activation did not cause the appearance of di- or multi-meric forms of the enzyme (the latter phenomenon was reported as a possible mechanism for activation of *Vicia faba* α -galactosidase [33]).

The results of the two above experiments, combined with evidence that the tyrosines remain unchanged (see above), indicate that oxidative activation does not involve intra- or inter-molecular dityrosine linking of the protein.

On the other hand, all the results from spectral and biochemical analysis favour the methionine as the target residue. The pH-dependence of the oxidative activation of α -galactosidase further supports this (Figure 1, inset). Only methionine reveals a similar dramatic increase in oxidation rate on pH decrease in a weakly acidic region [19].

DISCUSSION

H_2O_2 , a well-known agent used for chemical modification of proteins [19,27,31], attacks thioether, indole, thiol, imidazole and phenol functional groups.

Several reports have demonstrated that methionine residues of proteins are more readily oxidized with H_2O_2 than cysteine or cystine. Tryptophan and tyrosine are much more resistant to peroxide attack and can be modified at relatively high pH, ranging from 8.0 to 10.0 [34]. We conclude that preferential oxidation of methionine in α -galactosidase from *T. reesei* is a logical outcome.

As indicated by experiments on protein protection from H_2O_2 by galactose (a competitive inhibitor), the target methionine is located close to or directly in the active site of the enzyme (Figures 1 and 8 and Table 3). Glucose, even when used at a higher concentration, had no protective effect.

As shown by experiments on galactose protection against mercurial compounds (Hg^{2+} and PCMB; see Figure 6), the single free thiol group on the enzyme is likewise associated with the active site. Inactivation of the enzyme by TSF (Figure 7) and strong inhibition by mercurial compounds (results not shown) inevitably leads to the conclusion that this group is important for catalysis.

Modification of a catalytically essential -SH group by H_2O_2 has been reported for glyceraldehyde-3-phosphate dehydrogenase [35], leading us to propose in earlier work [28] that this particular group accounted for oxidation-induced activation. The present studies on α -galactosidase show that this group remains unmodified during protein activation.

Confirmation of this is provided by the following observations. (1) Quantitative titration of the thiol group gave identical results for the native and activated forms of α -galactosidase (Figure 6). (2) The native and oxidatively activated enzymes showed similar behaviour in the experiments with excess cysteamine after the addition of TSF: inactivation of the protein was followed by 50% re-activation (Figure 7). (3) The presence of PHMB, PCMB or a molar equivalent of $HgCl_2$, all of which bind the -SH group, enhanced activation but inhibited subsequent inactivation (Figure 1).

H_2O_2 is a convenient oxidant as it can be easily removed immediately after reaction, preventing it from having further effects on the protein or substrate. Other commonly used oxidants, for example $NaIO_4$, are less suitable because they cannot be removed. One more factor against the use of periodate is that α -galactosidase is a glycoprotein and its treatment with periodate would result in destruction of the carbohydrate moiety [36]. This, in turn, may induce changes in physical, chemical and, possibly, enzymic properties of the protein [20], hampering interpretation of the experimental data.

In our experiments, oxidation of methionine by non-specific agents, such as H_2O_2 and $NaIO_4$, proceeded as far as methionine sulfoxide. Subsequent formation of methionine sulphone requires longer time periods and harsher conditions [25], inevitably leading to inactivation of the enzyme as the result of oxidation of the thiol group. Therefore we could not examine α -galactosidase properties after conversion of methionine into methionine sulphone.

That only about one of five methionine residues on the native α -galactosidase is oxidized by H_2O_2 during the short (1.5 h) incubation could probably be attributed to steric hindrance. Protein denaturation renders the remaining intact methionines more accessible. After denaturation with guanidine, two methionines per protein molecule became oxidized (Tables 2 and 3), whereas acid denaturation at pH 0.7 and 40 °C resulted in oxidation of four methionine residues. Prolonged (3 days) oxidation of the native enzyme gave approximately three oxidized methionine residues, as judged by differential NMR (Figure 8). For comparison, oxidation of native human α_1 -proteinase inhibitor results in modification of two of ten methionines [37], and in human serum transferrin two of a total of eight methionines are modified [38].

The mechanism by which the properties of α -galactosidase are changed during conversion of the catalytically important methionine into methionine sulfoxide remains largely unelucidated. However, it is evident that, on oxidation of methionine to methionine sulfoxide, the polarity of the residue is increased, rendering it more hydrophilic. This may cause local conformational changes in or near the active site. Indeed, the active site becomes, in general, more accessible to any agents, e.g. catalytic poisons, substrates, inhibitors. This is shown by a higher rate of reaction of the modified enzyme with mercurial compounds (Figure 6) or TSF and thiols (Figure 7).

There are several methods of enzyme activation. For glycosidases such as β -xylanase from *Irpex lacteus*, enhanced activity is caused by the action of Ca^{2+} [39]. Neutral α -mannosidase isolated from Japanese quail oviduct is activated by Co^{2+} [40]. An interesting example of oxidative activation is activation of galactose oxidase in the presence of H_2O_2 and plant peroxidase: dityrosine is formed, leading to stabilization of the active conformation [31]. Pridham and Dey [33] described activation of α -galactosidase from mature *V. faba* seeds both *in vivo* and *in vitro* when 38 kDa monomers of the enzyme were self-associated into tetramers. K^+ ions also increased the activity of the enzyme. The multimers found *in vivo* were proposed to be in dynamic equilibrium with the monomers, such a mechanism being regarded as one of the methods of autoregulation of enzyme activity during maturation and germination of *V. faba* seeds.

However, our investigation of the possible effect of HRP on oxidative activation, as well as the search for protein fragments of di- or multi-meric forms of α -galactosidase in the samples of activated enzyme, gave negative results (Figure 10).

We may have identified a new (or at least previously undescribed) way of increasing enzyme activity via chemical modification of a single methionine in the active site. Earlier reports on methionine oxidation in proteins and enzymes showed that oxidation was generally without effect on activity [36] or induced complete inactivation, as was the case for apotransferrin [38]. Perhaps at present, statements about the unique character of the observed effect would be premature. Further studies are required on other carbohydrates with methionine in the active site, such as β -galactosidase from *E. coli* [13].

In conclusion, the discovery of the catalytically important methionine in α -galactosidase from *T. reesei* may be useful for research on the active site of the protein. As a part of this

programme, we will attempt to establish whether there is a spatial contact between active methionine and active cysteine or tryptophan by comparing two-dimensional NOESY NMR spectra of the native and activated α -galactosidases.

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