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Reactivity of galactose oxidase

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Abstract

Recent studies on reactions of the two-equivalent Cu^{II}/tyrosyl radical containing enzyme galactose oxidase (GOase) from *Fusarium* NRRL 2903 are referred to in this report. Two $GOase_{ox}$ active-site acid dissociation pK_a values have been determined by UV–vis spec-

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trophotometry, and are 5.7 (coordinated H_2O) and 7.0 (protonated Tyr-495). The active enzyme ($GOase_{ox}$) catalyses the oxidation of the primary alcohols $RCH_2OH + O_2 \rightarrow RCHO + H_2O_2$, where previous studies with five different substrates are extended to include saturation kinetics for D-Galactose and D-Raffinose. Two competing steps, $GOase_{ox} + RCH_2OH \rightarrow GOase_{red}H_2 + RCHO$ (k_1) and $GOase_{red}H_2 + O_2 \rightarrow GOase_{ox} + H_2O_2$ (k_2) are observed. Rate constants k_1 are dependent on pH, whereas k_2 is independent of pH in the range 5.5–9.0. The k_2 behaviour suggests that the two protons required to bring about the $O_2 \rightarrow H_2O_2$ conversion are provided by the protonated form $GOase_{red}H_2$. Michaelis–Menten kinetics allow K_m ($= 1/K_{bind}$) and k_{cat} (catalytic turnover) to be determined, where K_{bind} M^{-1} values are small for D-Galactose (6.7) and D-Raffinose (14.3), in keeping with the enzyme being extracellular. Pulse radiolysis studies on $GOase_{semi}$ with $CO_2^{\bullet -}$ (which is unable to provide protons) are also described. These indicate that, after initial reduction to the Cu^I state ($GOase_{red}$), a spontaneous decay of the unstable product occurs with formation of a disulfide radical anion ($RSSR^{\bullet -}$) detected by its absorbance at 450 nm. Slow decay of the disulfide radical is observed in a further step. Evidence obtained suggests that the spontaneous decay of the tyrosyl radical of $GOase_{ox}$ also involves the disulfide. As a means of modelling substrate binding, NCS^- substitution at the Cu^{II} active site of $GOase_{ox}$ has been investigated. Dependence on pH is again observed and at 25°C, pH 7.0 (10 mM phosphate), $K = 0.5 \times 10^3$ M^{-1}, with forward rate constant $k_f = 1.1 \times 10^4$ M^{-1} s^{-1}, $I = 0.100$ M (NaCl).

Keywords: Galactose oxidase; Acid dissociation processes; Pulse radiolysis; Substitution with NCS^-

1. Introduction

Galactose oxidase (GO; EC 1.1.3.9) is a single Cu^{II} tyrosyl radical containing enzyme with a molecular weight of 68.5 kDa (639 amino acids) [1–3]. It catalyses the stereospecific two-electron oxidation of a wide range of primary alcohols (RCH_2OH), including sugars and polysaccharides, to the corresponding aldehydes under aerobic conditions. Substrate oxidation is coupled with the reduction of molecular oxygen to hydrogen peroxide (Eq. (1)).

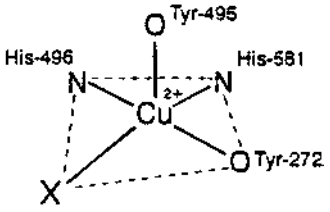
$$RCH_2OH + O_2 \rightarrow RCHO + H_2O_2 \quad (1)$$

The three oxidation states of galactose oxidase are here referred to as $GOase_{ox}$ (Cu^{II}, $Tyr^{\bullet}$), $GOase_{semi}$ (Cu^{II}, Tyr), and $GOase_{red}$ (Cu^I, Tyr). The $GOase_{semi}$ form is not involved in the catalytic cycle (Eqs. (2) and (3)) [4]:

$$RCH_2OH + GOase_{ox} \rightarrow RCHO + GOase_{red} \quad (2)$$

$$GOase_{red} + O_2 \rightarrow GOase_{ox} + H_2O_2 \quad (3)$$

but is important for a full understanding of the properties of the enzyme. Three X-ray crystal structures with a resolution down to 1.7 Å have shown that the Cu^{II} has a square-pyramidal geometry, and is coordinated by Tyr-272,



Tyr-495 (axial ligand), His-496 and His-581 with an additional exogenous ligand X, which is believed to be the substrate binding site. Structures reported are with X in turn acetate [5,6], azide [7], and water (H_2O or OH^-), [5]. Extended X-ray absorption fine structure (EXAFS) data have indicated that there are no significant differences in the coordination sphere of the Cu^{II} in $GOase_{semi}$ and $GOase_{ox}$ [8]. In the active $GOase_{ox}$ form the enzyme contains a coordinated tyrosyl radical at Tyr-272 [9–11]. Unusual features of Tyr-272 are a thioether link in the *ortho*-position of the phenolate oxygen formed by the covalent binding of the S-atom of Cys-228, which is overlayed by the indole π-system of Trp-290 [12]. It is believed that both features help stabilise the radical formed at Tyr-272 [12], which has a reduction potential at pH 7.5 of 400 mV (vs. NHE) [13], the smallest value so far observed for a tyrosyl radical ($Tyr^{\bullet}$).

Despite these stabilising effects a spontaneous decay of the $Tyr^{\bullet}$ of $GOase_{ox}$ occurs over ~ 3 h [14]. Other studies referred to are the kinetics of the catalytic cycle (Eqs. (2) and (3)), the determination of pK_a values for $GOase_{ox}$, the instability of unprotonated $GOase_{red}$ generated by pulse radiolysis, and a modelling of substrate binding using NCS^-. A recent report on $GOase_{ox}$ studies in 50 mM phosphate buffer by Whittaker et al. [15] contains misleading statements which are attributed to effects of phosphate binding [16].

2. Experimental

2.1. Enzyme source

Wild-type (WT) *Fusarium* NRRL2903 GOase was isolated from an *Aspergillus nidulans* expression system using procedures already described [17]. The enzyme is strongly basic (pI = 12) with a charge balance from Glu, Asp (negative) and Lys, Arg (positive) residues of $+9$ at pH ~ 7 [18]. Final protein purification was carried out on a phosphocellulose column. The purity of samples was confirmed by fast protein liquid chromatography (FPLC; Pharmacia), using a Mono-S cation-exchange column, when a single peak was obtained.

Total protein concentrations were determined from the absorbance at 280 nm ($\epsilon = 1.05 \times 10^5\ M^{-1}\ cm^{-1}$) [19], which is independent of oxidation state and pH. The enzyme used in this work had $\sim 70\%$ Cu content, and $\epsilon = 8600\ M^{-1}\ cm^{-1}$ for $GOase_{ox}$ (pH 7.0) at the 450 nm peak. The buffers Mes, phosphate, Ches and Caps

have negligible absorbance at 280 nm. Corrections were made for the absorbance of lutidine at < 300 nm.

2.2. Buffers

The following buffers (all from Sigma) were used: 2-[*N*-morpholino] ethanesulfonic acid (Mes, pK_a 6.1, pH 5.5–6.7); 2-6-dimethylpyridine (Lutidine, pK_a 6.75, pH 6.0–8.0); sodium hydrogen phosphate (pK_a 7.0, pH 5.7–8.0); 2-[*N*-cyclohexylamino]-ethane-sulphonic acid (Ches, pK_a 9.3, pH 8.6–10.0); and 3-[cyclohexylamino]-1-propanesulfonic acid (Caps, pK_a 10.4, pH 9.7–11.1). Buffer solutions were made up on the same day of use or stored overnight at 4°C.

2.3. Other reagents

Potassium hexacyanoferrate(III) trihydrate, $K_3[Fe(CN)_6]$ (Sigma), potassium hexacyanoferrate(II) $K_4[Fe(CN)_6]\cdot 3H_2O$ (BDH, Analar), sodium thiocyanate NaSCN (Fluka Analar), D-Galactose (Sigma), and D-Raffinose (Sigma), were used as supplied without further purification.

2.4. Procedures for solutions studies

Enzyme as isolated is a mixture of $GOase_{ox}$ and $GOase_{semi}$ (typically 95% $GOase_{semi}$) often referred to as native enzyme. To prepare solutions of $GOase_{ox}$ or $GOase_{semi}$, 2 ml samples of native enzyme (3–5 mg per ml) stored at -80°C were dialysed against the desired buffer (2 l) for ~ 12 h at 4°C. To convert to the oxidised or semi form a 100-fold excess of $[Fe(CN)_6]^{3-}$ or $[Fe(CN)_6]^{4-}$ was added. After 2–3 min the inorganic reagent was removed by either ultra-filtration using an Amicon PM-30 membrane, or by passing the solution down a small desalting column (0.8×20 cm, BioRad P-6 DG resin).

Solutions were made up using 10 mM buffer and adjusted to $I = 0.100 \pm 0.001$ M (NaCl), except for those runs in 50 mM phosphate, which have $I = 0.120$ M at pH ~ 7.0. Distilled water was further purified by passing it through a de-ioniser column (Sanyo) to give a conductivity of ~ 10 μS cm^{-1}. All studies were carried out at 25.0 ± 0.1°C, except the pulse radiolysis experiments which were at 22 ± 1°C. Conventional spectrophotometry was carried out on Shimadzu 2101 PC or Perkin-Elmer Lambda 9 instruments. Reactions of $GOase_{ox}$ with substrate in the presence of O_2 were monitored using an Applied Photophysics SX-17MV Stopped-Flow Reaction Analyser at a fixed wavelength (450 nm). The dioxygen content of reactant solutions was determined using a Beckman Model 0260 Oxygen Analyser with a thermally compensated 39556 electrode. Absorbance–time traces were fitted to the catalytic reaction scheme (2)–(3) using the Applied Photophysics Global Analysis Software package. Kinetic studies on the reaction of NCS^- with $GOase_{ox}$ were also by the stopped-flow method.

2.5. Procedures for pulse radiolysis

All pulse radiolysis (PR) experiments were carried out on a Van de Graaff accelerator at the Cookridge Research Centre, Leeds using a multipass cell (6.9 cm path length) and a 2.5 MeV ($\sim 4 \times 10^{-16}$ J) beam of electrons. Pulse lengths were 0.6 μs and the yield of reducing radicals generated by the pulse were determined procedures [20]. All runs were performed under rigorous anaerobic conditions in N_2O-saturated phosphate buffer containing 0.010 M sodium formate ($I = 0.100$ M). Under these conditions the primary products were converted to $CO_2^{\bullet -}$ as the only reducing radical. Small amounts of the concentrated GOase (~ 0.2 mM) were added to the N_2-saturated buffer solution to give a final GOase concentration of ~ 10 μM. Reactions were monitored in the range 380–510 nm with $[CO_2^{\bullet -}] < 0.6$ μM. Absorbance versus time traces were recorded on a Gould 4072 100 MHz Transient Digitiser, which was used in conjunction with a Jun SPARC Station IPC computer for storage and processing. The PR traces were subject to first-order kinetic analysis.

3. Results and discussion

3.1. Acid dissociation constants for $GOase_{ox}$

Titration of a solution of $GOase_{ox}$ (15 μM), pH ~ 4.0 (no buffer), with 0.10 M NaOH was monitored by recording UV–vis spectra. Chemical changes were rapid, and it was possible to complete titrations within 20 min. From absorbance changes at fixed wavelengths $pK_{1a} = 5.7$ (isosbestics at 466 and 549 nm), and $pK_{2a} = 7.0$ (no isosbestics), were obtained [16]. Absorbance changes at a single wavelength do not clearly separate the two processes [14]. With solutions of the $GOase_{ox}$ Tyr495Phe variant pK_{2a} is not detected [16]. When the substrate binding site X is occupied by an exogenous group other than H_2O no pK_{1a} is observed. The two processes are defined as in Eqs. (4) and (5):

$$GOase_{ox}(H_2O) \overset{K_{1a}}{\rightleftarrows} GOase_{ox}(OH) + H^+ \quad (4)$$

$$GOase_{ox}(Tyr\text{-}495H^+) \overset{K_{2a}}{\rightleftarrows} GOase_{ox}(Tyr\text{-}495) + H^+ \quad (5)$$

with acid dissociation of H_2O at the exogenous site indicated in Eq. (4), and of protonated Tyr-495 in Eq. (5).

3.2. Catalytic process

Reactions of $GOase_{ox}$ with five RCH_2OH substrates in the presence of dioxygen have been studied by stopped-flow spectrophotometry [14]. The substrates were as follows: D-galactose (I) and 2-deoxy-D-galactose (II) (monosaccharides); methyl-β-D galactopyranoside (III) (glycoside); D-Raffinose (IV) (trisaccharide); and dihydroxyacetone (V). The decay of $GOase_{ox}$ was monitored at 450 nm, where reactions

terminate on depletion of O_2. Each trace was fitted to the competing reactions (6)–(7).

$$GOase_{ox} + RCH_2OH \xrightarrow{k_1} GOase_{red}H_2 + RCHO \quad (6)$$

$$GOase_{red}H_2 + O_2 \xrightarrow{k_2} GOase_{ox} + H_2O_2 \quad (7)$$

At pH 7.5, rate constants 10^{-4} k_1 M^{-1} s^{-1} for the reactions of $GOase_{ox}$ with (I) (1.19), (II) (1.07), (III) (1.29), (IV) (1.81), (V) (2.94) were determined. On decreasing the pH to 5.5 values of k_1 decreased by about one half. The effects of pK_{1a} and pK_{2a} give single 'apparent' acid dissociation pK_a values in the range of 6.6–6.9 in accordance with values from UV–vis spectrophotometry already reported [14].

The catalytic cycle with D-Galactose and D-Raffinose as substrates for $GOase_{ox}$ in the presence of O_2 has been further studied with substrate concentrations ranging from 2 to 100 μM. Reactant solutions at pH 7.0, buffered in 10 mM phosphate, were compared with those in 50 mM phosphate, when the phosphate binds at the exogenous site. Rate constants for the catalytic reaction in 10 mM buffer are generally 2–15% larger than those in 50 mM phosphate. It therefore appears that both substrates are able to displace the phosphate quite readily, and preliminary results indicate rapid competitive inhibition by the phosphate. First-order rate constants (k_{1obs}) over the extended substrate range give a non-linear dependence on substrate concentration (Fig. 1), which exhibits saturation kinetics and conforms to the Michaelis–Menten equation (Eq. (8)),

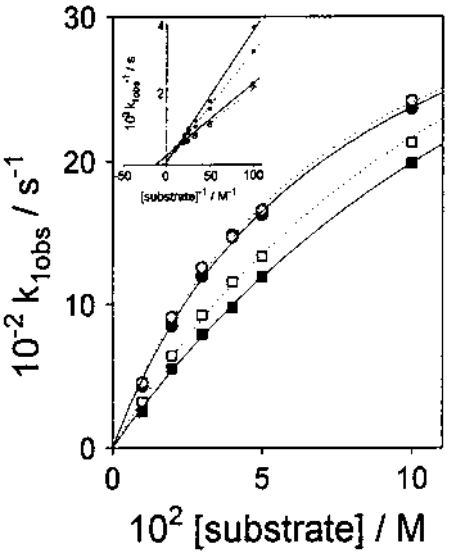


Fig. 1. Plot of the first-order rate constants k_{1obs} (25°C) for the catalytic reactions of $GOase_{ox}$ with D-Galactose (□) and D-Raffinose (○) in 10 mM phosphate, and for the enzyme in 50 mM phosphate (■ and ●, respectively), pH 7.0, $I = 0.100$ M (NaCl), illustrating the saturation kinetic behaviour.

$$k_{1obs} = \frac{k_{cat}[\text{substrate}]}{K_m + [\text{substrate}]} \quad (8)$$

where K_m is the Michaelis constant ($1/K_{bind}$) and k_{cat} is the catalytic turnover number of the enzyme. This non-linear dependence is also illustrated by the corresponding Lineweaver–Burk plots [21].

The values obtained for K_m and k_{cat} for D-Galactose are 0.15(1) M and 5500(300) s^{-1} in 10 mM buffer, and 0.20(2) M and 5900(400) s^{-1} in 50 mM buffer, while those for D-Raffinose are 0.07(1) M and 4200(200) s^{-1} in 10 mM buffer, and 0.09(1) M and 4200(200) s^{-1} in 50 mM buffer, respectively. From K_m, values of K_{bind} M^{-1} in 10 and 50 mM phosphate are 6.7 and 5.0 for D-Galactose, and 14.3 and 11.1 for D-Raffinose, which compare with earlier values of 4.0 and 14.1 D-Galactose and D-Raffinose, respectively [17,22]. Such small values reflect the stereospecific nature of the reaction. The precise substrate(s) for GOase have not been identified with certainty, but are likely to be the primary alcohol groups of carbohydrate molecules. Since the enzyme is extracellular high K_{bind} value are not essential.

Rate constants k_2 (1.01×10^7 M^{-1} s^{-1}) on the other hand are independent of substrate and pH (5.5–8.6). This has led to the suggestion that protons required for the $O_2 \rightarrow H_2O_2$ change are provided by doubly-protonated $GOase_{red}H_2$. Protonation of the active site is an outcome of the $RCH_2OH \rightarrow RCHO$ redox change, with the two tyrosine ligands as possible sites for protonation. A coordination number five is unlikely to be retained by the d^{10} Cu^I of $GOase_{red}$ and a coordination number three (for example) is much more reasonable. Since the Tyr495Phe variant has no catalytic activity, and the protonated Tyr-495 of WT $GOase_{ox}$ retains appreciable activity, it seems unlikely that full dissociation of the Tyr-495 occurs.

3.3. Pulse radiolysis

Studies on the reduction of $GOase_{semi}$ with the formate radical $CO_2^{\bullet -}$ at pH 7.00 indicate three separate clearly defined stages [15]. The mechanism proposed (Eqs. (9)–(11)), implicates a disulfide bridge. In these equations $GOase_{semi}$ is written as $Cu^{II}\cdots RSSR$.

$$Cu^{II}\cdots RSSR + CO_2^{\bullet -} \xrightarrow{k_3} Cu^{I}\cdots RSSR + CO_2 \quad k_3 > 10^9 \ M^{-1} \ s^{-1} \quad (9)$$

$$Cu^{I}\cdots RSSR \xrightarrow{k_4} Cu^{II}\cdots RSSR^{\bullet -} \quad k_4 = 83 \ s^{-1} \quad (10)$$

$$Cu^{II}\cdots RSSR^{\bullet -} \xrightarrow{k_5} Cu^{II}\cdots RSSR \quad k_5 = 0.17 \ s^{-1} \quad (11)$$

An initial rapid reduction of $GOase_{semi}$ with $CO_2^{\bullet -}$ (-1.9 V) is observed ($k_3 > 10^9$ M^{-1} s^{-1}). However, $CO_2^{\bullet -}$ is not a provider of protons, and the reduction results in the formation of an unstable form of $GOase_{red}$ quite different from $GOase_{red}$ produced during the catalytic cycle with substrate. The second stage corresponds to intramolecular electron transfer (ET) from the Cu^I of the metastable $GOase_{red}$ to a nearby disulfide bridge with generation of the radical ($k_4 = 83$ s^{-1}). This can be regarded as an escape route for the unwanted electron. The disulfide involved is most likely Cys515-Cys518, nearest S-atom at 10.2 Å from the Cu^I. A second disulfide bridge is much further away (~ 40 Å). The disulfide radical anion ($RSSR^{\bullet -}$) is identified by its UV–vis-absorption spectrum, with peak maximum at 450 nm

($\epsilon = 8 \times 10^3\ M^{-1}\ cm^{-1}$). An identical absorbance spectrum has been observed for the $RSSR^{\bullet -}$ radical formed in studies on metallothionein [22]. In the case of azurin, when the two cysteines of the disulfide are further apart (positions 3 and 26), a value of 410 nm has been reported [22]. A slow decay of the disulfide radical follows ($k_5 = 0.17\ s^{-1}$), in a process which is not at present fully understood. There is no evidence for any direct reaction of the $CO_2^{\bullet -}$ radical with the disulfide group.

3.4. Spontaneous decay of $GOase_{ox}$

Auto-redox conversion of the $GOase_{ox}$ and $GOase_{semi}$ forms can be monitored by UV–vis spectrophotometric changes. At pH 7.5 the process requires at least 2 h. Spectra converge to an equilibrium mix close to 95:5 of $GOase_{semi}$ to $GOase_{ox}$ [14]. First-order equilibrium rate constants from absorbance changes at 450 or 810 nm are $\sim 1.8 \times 10^{-4}\ s^{-1}$; chemical modification of the Cys515-Cys518 disulfide suggests that this group is involved.

3.5. Reaction of $GOase_{ox}$ with NCS^-

Binding of NCS^- to $GOase_{ox}$ serves as a model for substrate binding [23]. The effects of pH have not previously been studied. A 1:1 uptake was demonstrated from UV–vis absorption changes. Equilibrium constants K are defined in Eq. (12).

$$GOase_{ox} + NCS^- \overset{K}{\rightleftarrows} GOase_{ox}(NCS^-) \tag{12}$$

At pH 7.0 (10 mM phosphate), $K(25°C) = 0.50 \times 10^3\ M^{-1}$, $I = 0.100$ M (NaCl). Both pK_{1a} and pK_{2a} have a controlling influence on K [22]. Values of K can also be expressed in terms of forward and back rate constants ($K = k_f/k_b$). For the same conditions using stopped-flow spectrophotometry $k_f = 1.11 \times 10^4\ M^{-1}\ s^{-1}$, when the separate effects of pK_{1a} and pK_{2a} are observed over the pH range 5.5 to 7.9. On replacing H_2O by OH^- (pK_{1a}) both K and k_f decrease in keeping with electrostatic repulsion between the OH^- and NCS^-. Deprotonation of Tyr-495 (pK_{2a}) gives smaller effects in both K (a decrease) and k_f (an increase).

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