

Mechanistic studies on the single copper tyrosyl-radical containing enzyme galactose oxidase

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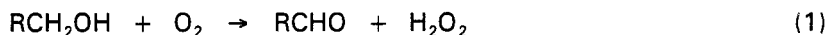
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Abstract: Studies on the single Cu protein galactose oxidase (68kDa; 639 amino acids) from *Fusarium* NRRL 2903 are described. The Cu is coordinated in a square based pyramid by Tyr-272, Tyr-495 (axial), His-496, His-581 and H₂O (the substrate binding site), and the enzyme functions as a 2-equivalent oxidase, O₂ → H₂O₂, with oxidation of primary alcohol substrates RCH₂OH to RCHO. The active enzyme has a coordinated tyrosyl (Tyr) free radical at Tyr-272, and along with the Cu^{II} to Cu^I redox change gives the required two-equivalent redox capacity. The three oxidation states are here written as GOase_{ox} (Cu^{II}, Tyr[•]), GOase_{semi} (Cu^{II}, Tyr), and GOase_{red} (Cu^I, Tyr). Protonation of Tyr-495 is an important part of the enzymic reaction. Studies described are consistent with a mechanism involving H-atom transfer from substrate RCH₂OH to Tyr[•] at Tyr-272.

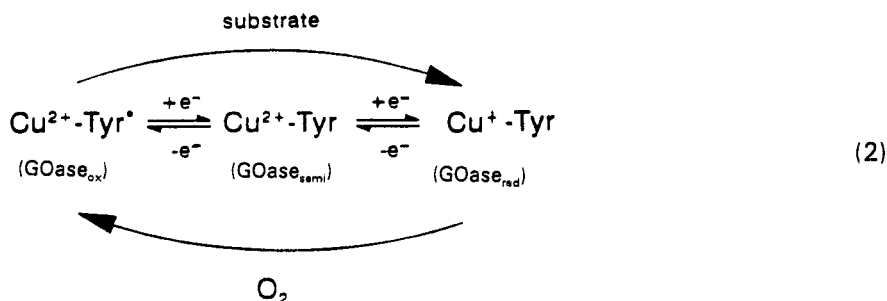
INTRODUCTION AND GENERAL PROPERTIES

Occurrence and Function

Galactose oxidase (GOase; EC 1.1.3.9) is a Type 2 single Cu enzyme secreted by a number of fungal species of which *Fusarium* NRRL 2903 formerly known as *Polyporus circinatus* and *Dactylium dendroides* has been the most extensively studied, (ref. 1-3). It serves as catalyst for the oxidation of a wide range of primary alcohols (RCH₂OH) including sugars, polysaccharides (in particular hexose derivatives), (ref. 2,4,5), in a two-equivalent oxidase action converting dioxygen to hydrogen peroxide.



The reaction is stereospecific, (ref. 6), and the enzyme for example reacts with D-galactose but not the L form. The oxidation states GOase_{ox} (Cu^{II}, Tyr[•]) and GOase_{red} (Cu^I, Tyr) are involved in this change, (2). The intermediate state GOase_{semi} (Cu^{II}, Tyr) although



not involved in the catalytic cycle is also important for a full understanding of the properties of the enzyme.

Structure

X-ray structure determinations of crystals obtained from the so-called 'native' mix of GOase_{semi}: GOase_{ox} in a ratio of approximately 95:5, (ref. 7), with in turn H₂O (ref. 8), acetate (ref. 8,9), and azide (ref. 10), at the substrate binding site on the Cu, have been determined at resolutions down to 1.7Å. EXAFS studies have indicated no significant change in structure between GOase_{semi} and GOase_{ox}, (ref. 11), and crystals grown at pH 4.5 in the presence of [Fe(CN)₆]³⁻ have the same structure as those obtained from solutions of the 'native' mix.

The Cu^{II} is close to the surface and has square pyramidal coordination to Tyr-272, Tyr-495 (axial), His-496, His-581 and H₂O, which is believed to be the substrate binding site, (ref. 8,9). Domain 2 provides three of the coordinating amino acids, and domain 3 the His-581. Unusual features are the thioether link formed by the covalent binding of the S-atom of Cys-228 at the ortho position of the phenolate of Tyr-272. The indole ring of Trp-290 overlays this bond and the adjacent phenolate, and is believed to help stabilise the radical formed at 272. From the outside the same indole ring controls entry to the active site.

Since there is only a single Cu atom, the possible involvement of a flavin mono-nucleotide or pyrolo-quinoline quinone cofactor was considered in earlier studies, as was the involvement of the Cu^{III}/Cu^I redox couple, in order to bring about a two-equivalent redox change, (ref. 12,13). However, in 1988, the Whittakers obtained evidence for the involvement of a tyrosyl radical, which is now a well established feature, (ref. 14-16).

Isolation of Enzyme

Wild-type *Fusarium* NRRL 2903 GOase was isolated from an *Aspergillus nidulans* expression system supplied by Dr. M. McPherson, Department of Biochemistry and Molecular Biology, University of Leeds, using procedures already described, (ref. 17). The Tyr495Phe and Trp290His variants were obtained in a similar manner.

Reduction Potentials

The reduction potential (E°) determined for the wild-type (WT) GOase_{ox}/GOase_{semi} couple at pH 7.5 is 400mV, which is the smallest value so far observed for a tyrosyl radical. On decreasing the pH the E° increases to 500mV at pH 5.5, (ref. 18). The Tyr495Phe variant has an E° of 415mV which does not vary with pH. This and other experiments indicate that protonation of Tyr-495 is influential on enzymic reactivity. It has not so far been possible to obtain a precise measure of the GOase_{semi}/GOase_{red} potential. Observations with [Co(terpy)₂]³⁺ (260mV) as an oxidant for GOase_{red} suggest that the E° may have a similar value.

Auto-redox of WT GOase_{ox} and GOase_{semi} forms

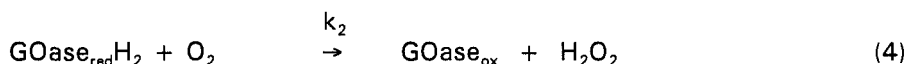
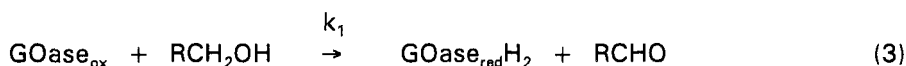
Auto-redox interconversion of the GOase_{ox} and GOase_{semi} can be monitored by UV-Vis spectrophotometric changes. At pH 7.5 the process requires at least 2 hours. Spectra converge to an equilibrium mix close to 95:5 of GOase_{semi} to GOase_{ox}, (ref. 7). First-order equilibration rate constants from absorbance changes at 450 or 810nm are $\sim 1.8 \times 10^{-4} \text{ s}^{-1}$. Some side effects were observed for reactions carried out in Hepes buffer (10mM) at times > 1 hour and other buffers (e.g. Mes and Lutidine) had to be used.

UV-Vis Spectrophotometric pK_a's

The Whittakers have assigned absorbance bands in the UV-Vis spectra of WT GOase_{semi} and GOase_{ox}, (ref. 16,19). In both cases dependencies on pH are observed from which pK_a values of GOase_{semi} (7.9) and GOase_{ox} (6.7) can be determined. No similar effects are observed for the Tyr495Phe variant and protonation is therefore at Tyr-495. The GOase_{red} has no visible range absorbance consistent with the Cu^I state.

Kinetic Studies of GOase_{ox} with Different Substrates in the Presence of O₂

The reactions of five different primary alcohols, RCH₂OH, have been studied, (ref. 7):- D-galactose (I) and 2-deoxy-D-galactose (II) (monosaccharides); methyl-β-D-galactopyranoside (III) (glycoside); D-raffinose (IV) (trisaccharide); and dihydroxyacetone (V). Reactant concentrations were [GOase_{ox}] (0.8 - 10 μM), RCH₂OH (1.0-6.0mM), and O₂ (0.14 - 0.29mM), with I = 0.100M(NaCl). The reactions monitored at 450nm by stopped-flow spectrophotometry terminated with the depletion of O₂. Each absorbance-time trace demonstrates complete recycling of GOase_{ox} until all the O₂ is used up, when permanent reduction to GOase_{red} is observed. Traces can be fitted to (3) and (4),

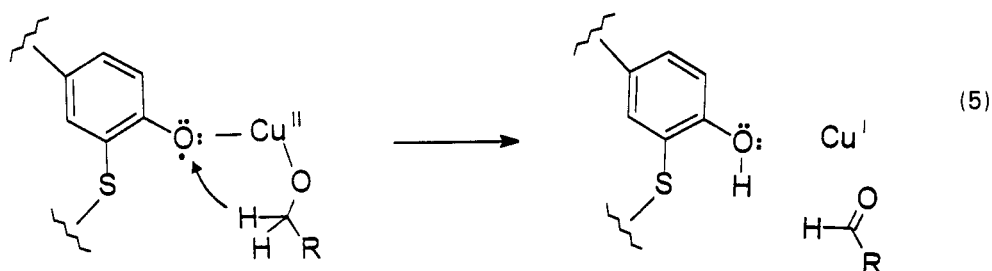


with GOase_{red}H₂ used here to indicate double protonation of the GOase_{red} product. At pH > 7.5 rate constants 10⁻⁴k₁ (25°C)/M⁻¹s⁻¹ are I (1.19), II (1.07), III (1.29), IV (1.81), V (2.94). On decreasing the pH to 5.5, k₁ values decreased by factors of as much as a half, and acid dissociation pK_a's in the range 6.6 - 6.9 are observed in agreement with the spectrophotometric value (6.7). There was no reaction with the Tyr495Phe variant. The rate constant k₂ (1.01 × 10⁷M⁻¹s⁻¹) is independent of substrate and pH in the range 5.5 to 9.0, suggesting that H⁺ (or H-atoms) for the O₂ → H₂O₂ conversion are provided by the GOase_{red} active site. The Cu^I of GOase_{red} is less extensively complexed, with a coordination number of two or three a strong possibility.

Mechanism of Enzymic Reaction

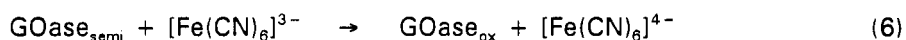
Bulk solvent acid dissociation pK_a values for alcohols are generally high, e.g. for methanol (15.54) and ethanol (16.00), and similar to those of water (15.74), (ref. 20). From studies on Zn^{II} and Cu^{II} macrocyclic triamine complexes with a pendant alcohol group attached, Kimura and colleagues, (ref. 21,22), have reported pK_a values for the coordinated alcohol close to 7.4 at 25°C. Proton release on binding RCH₂OH to GOase_{ox} may occur therefore. No spectrophotometric changes consistent with acid dissociation of H₂O at the substrate binding site of GOase_{ox} have been observed, and the long Cu-O (H₂O) of ~2.80Å from X-ray crystallography, (ref. 8), suggests that H₂O may not as readily dissociate as in the case of aqua Cu^{II} complexes (pK_a's 7.3 - 8.0), (ref. 23).

We conclude that protonation of Tyr-495 and acid dissociation of the RCH₂OH occur as independent processes. The observation that protonated Tyr-495 retains > 50% enzymic reactivity at low pH's, whereas Tyr495Phe gives no enzymic reaction, suggests that GOase_{ox} with a protonated Tyr-495 remains functional. The mechanism has previously been discussed in terms of a stepwise radical mechanism with the ketyl radical R·CH₂O⁻ as an intermediate. However since there is no strong evidence for the existence of R·CH₂O⁻ we suggest a concerted mechanism as in (5) involving the Tyr-272 radical.



Kinetic Studies with Inorganic Complexes as Redox Partners

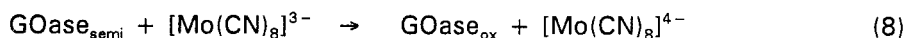
Redox interconversions between the GOase_{semi} (Cu^{II}) and tyrosyl radical containing GOase_{ox} (Cu^I, Tyr) oxidation states have been studied, (ref. 18). The inorganic complexes [Fe(CN)₆]³⁻ (410mV), [Co(phen)₃]³⁺ (370mV), [W(CN)₈]³⁻ (530mV) and [Co(dipic)₂]⁻ (362mV) (E^o values vs nhe; dipic = 2,6-dicarboxylatopyridine) were used as oxidants for GOase_{semi}, and [Fe(CN)₆]⁴⁻ and [Co(phen)₃]²⁺ as reductants for GOase_{ox}, e.g. (6) and (7),



with the inorganic complex in large >10-fold excess, so that reactions proceed to >90% completion. On oxidation of GOase_{semi} a radical is generated at the coordinated phenolate of Tyr-272 to give GOase_{ox}. The reactions are very sensitive to pH, or more precisely to pK_a values of GOase_{semi} and GOase_{ox}, and the charge on the inorganic reagent. For example with [Fe(CN)₆]³⁻ as oxidant the rate constant (25°C)/M⁻¹s⁻¹ of 0.16 × 10³ (pH ~9.5) increases to 4.3 × 10³ (pH ~ 5.5), while for [Co(phen)₃]³⁺ a value of 4.9 × 10³ (pH ~9.5) decreases to 0.04 × 10³ (pH ~ 5.5), I = 0.100M (NaCl). From the kinetics a single GOase_{semi} acid dissociation process, pK_a = 8.0 (average), compares with 7.9 from UV-Vis spectrophotometric studies. No comparable kinetic or spectrophotometric pH dependencies are observed with the Tyr495Phe variant, indicating the axial Tyr-495 as the site of protonation, (ref. 18).

Properties of the Trp290His Variant

The indole ring of Trp290 has been identified as having an important role in restricting entry to the substrate binding (Cu) site of galactose oxidase via a short ~8Å access pocket/channel. It also overlays and helps stabilise the radical-forming Cu-coordinated Tyr-272. In these studies the effect of replacing Trp-290 by the less bulky His residue was explored at 25°C, I = 0.100M (NaCl), and different effects quantified, (ref. 25). Interactions with buffers, not observed in the case of wild-type (WT) GOase, have been investigated by UV-Vis spectrophotometry on the non-radical GOase_{semi} (Cu^{II} form) of the Trp290His variant. Equilibrium constants K_{eq}/M⁻¹ from absorbance changes at ~635nm are for 1:1 interactions with the OH-containing buffers H₂PO₄⁻ (231), HEPES (43) and Tris (202), concentrations 0-60mM. It has not been established whether the buffer coordinates to the Cu, but it would be surprising if this were not the case from the magnitude of the changes observed. No similar interactions are observed with Mes, Lutidine and Ches, when significantly different UV-Vis spectra with no peak at ~635nm are obtained, (ref. 25). At pH 7.5 the reduction potential for the Trp290His GOase_{ox}/GOase_{semi} couple is 730mV, which compares with 400mV for WT GOase. Consistent with the 730mV value the GOase_{semi} form is not oxidised with [Fe(CN)₆]³⁻ (410mV) or [W(CN)₈]³⁻ (530mV), and much stronger oxidants such as [Mo(CN)₈]³⁻ (800mV) and [IrCl₆]²⁻ (890mV) are required, (8).



Moreover the GOase_{ox} product is unstable and spontaneously decays within 20min with re-formation of GOase_{semi}. From changes in UV-Vis spectra with pH Trp290His GOase_{semi} a pK_a of 6.9 is obtained, and rate constants for the oxidation of GOase_{semi} with [Mo(CN)₈]³⁻ are dependent on this same pK_a. The latter compares with 7.9 for WT GOase_{semi} and is assigned also as protonation of Tyr-495, (ref.25).

Summary

The single Cu active site of GOase, made two-equivalent by formation of a radical Tyr at Tyr-272, shows quite unique properties. At pH 7.5 the reduction potential (E°) for the Tyr of WT enzyme is small at 400mV. The indole of Trp-290 overlays and helps stabilise the Tyr. On replacing the Trp-290 by His the E° increases to 730mV, and GOase_{ox} undergoes decay over ~20min. Redox reactions of WT GOase_{semi} with inorganic complexes are very sensitive to charge and protonation of the GOase at Tyr-495. One particularly interesting property associated with the redox changes is the ability of the Cu to change its coordination, and hold two protons at the GOase_{red} active site, thus facilitating the O₂ → H₂O₂ redox change.

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