Reactivity of the Cu^{II} and Tyrosyl Free-Radical Active Site of the Enzyme Macromolecule Galactose Oxidase (GOase)

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SUMMARY: Studies on the single Cu enzyme galactose oxidase (68kDa; 639 amino acids) from fungal *Fusarium* NRRL 2903 are considered. The enzyme reacts as a $2e^-$ oxidase and catalyses the oxidation of primary alcohols such as D-galactose, $RCH_2OH + O_2 \rightarrow RCHO + H_2O_2$. The Cu^{II}, ~8Å from the surface, is accessed by a channel which imposes stereospecific selectivity (e.g. no reaction is observed with L-galactose). The Cu^{II} is coordinated by Tyr-272, Tyr-495, His-496, His-581 and H_2O (the substrate binding site) in a square pyramidal geometry. The active enzyme has a tyrosyl ($Tyr^{\cdot}$) radical at 272, which together with the Cu^{II} gives the required two-equivalent redox activity. The active form of the enzyme, $GOase_{ox}$,, is reformed by the $2e^-$ oxidation $O_2 \rightarrow H_2O_2$. Acid-base properties of the coordinated Tyr-495 and H_2O, and binding of NCS^-, N_3^-, $CH_3CO_2^-$ and $H_2PO_4^-$ in place of H_2O are considered

INTRODUCTION

Galactose oxidase (GOase; EC 1.1.3.9) is obtained from fungal sources, e.g. *Fusarium*, also referred to as Canadian wood-rot.[1,2] Previous papers on the reactivity are listed.[3-5] The three oxidation states of GOase are defined in (1), where the combined $2e^-$ oxidase activity of the Cu^{II} and tyrosyl radical ($Tyr^{\cdot}$) is indicated.

$$\begin{array}{ccccc} & \overset{+e^-}{\rightleftharpoons} & & \overset{+e^-}{\rightleftharpoons} & \\ Cu^{II}\text{ - }Tyr^{\cdot} & \underset{-e^-}{} & Cu^{II}\text{ - Tyr} & \underset{-e^-}{} & Cu^{I}\text{ - Tyr} \\ GOase_{ox} & & GOase_{semi} & & GOase_{red} \end{array} \qquad (1)$$

X-ray crystal structures at resolutions down to 1.7Å have demonstrated that the Cu^{II} has a square-pyramidal geometry, with Tyr-272 (radical forming), His-496, His-581 and the exogenous ligand X occupying the square-planar positions, and Tyr-495 as the axial ligand. In the first of these structures acetate buffer was incorporated at position X.[6] Crystals obtained from Pipes buffer at pH 7.0 have OH^-/H_2O at X,[6] and in a further study azide is coordinated at

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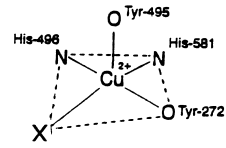
X.[7] From exafs data it has been concluded that there is no significant change in the active site structure of $GOase_{ox}$ and $GOase_{semi}$.[8] In the catalytic cycle the $2e^-$ reduction of $GOase_{ox}$ with the primary alcohol RCH_2OH, (2), is followed by the reaction with O_2, (3).[4,9]



$$RCH_2OH + GOase_{ox} \xrightarrow{k_1} RCHO + GOase_{red} \quad (2)$$

$$GOase_{red} + O_2 \xrightarrow{k_2} GOase_{ox} + H_2O_2 \quad (3)$$

The $GOase_{semi}$ state defined in (1) is not involved in the catalytic cycle. In (3) H_2O_2 is released, and may also play a part in processes such as lignin degradation. Studies described are largely with $GOase_{ox}$. The properties of $GOase_{semi}$ are also of interest. $GOase_{red}$ is difficult to isolate, and little is known about its properties.

It is likely that RCH_2OH and O_2 bind at more or less the same site, although the coordination number of the Cu^I state in $GOase_{red}$ will decrease, and it is not certain which groups are retained.[4] The alternate binding of alcohol and O_2 is sometimes referred to as a 'ping-pong' mechanism. In the case of the D-galactose and D-raffinose substrates enzyme kinetic studies have allowed k_{cat} and K_M to be determined.[9]

Acid Dissociation Constants for $GOase_{ox}$ and $GOase_{semi}$

Titrations of $GOase_{ox}$ and $GOase_{semi}$ ($15\mu mol\ dm^{-3}$) solutions, pH ~4.0 (no buffer) with 0.10M NaOH have been monitored by UV-Vis spectrophotometry.[10] Chemical changes are rapid, and it was possible to complete titrations within 20 min. Checks were carried out to ensure that no denaturation had occurred. Thus at the end of each titration the pH was adjusted to the starting pH and the absorbance checked. For $GOase_{ox}$ isosbestic points at 466 and 549nm were observed for the pH range 4.0-6.5, and from absorbance changes at a fixed wavelength $pK_{1a} = 5.7$. Absorbance changes at higher pH's give no isosbestics and $pK_{2a} = 7.0$. Similar procedures were used for $GOase_{semi}$ and for the Tyr495Phe variant of $GOase_{ox}$. The latter gives no pK_{2a}, and on this evidence pK_{2a} is assigned as the acid dissociation of Tyr495 H^+. The axial Cu-O(Tyr-495) bond of wild-type GOase is long due to Jahn-Teller distortions. It is not clear whether the Cu-O bond cleaves on protonation. Other pK_a values obtained from variations in K and k_f with pH are also entered in Table 1. Variations of rate constants with pH for four different oxidants

$[Fe(CN)_6]^{3-}$, $[Co(phen)_3]^{3+}$, $[W(CN)_8]^{3-}$ and $[Co(dipic)_2]^-$ for $GOase_{semi}$ also give pK_{2a} values.[3] From data at the lower pH's in the latter studies trends corresponding to pK_{1a} were not clearly defined. Some care is required in working at the lower pH's, when longer times are required for experimental routines, as the enzyme is less stable below pH 6.

Table 1: Summary of acid dissociation pK_a values (25°) for different wild-type (WT) and variant galactose oxidase (GOase) states, I = 0.100M (NaCl).

Enzyme	Method	pK_{1a}	pK_{2a}	Reference
WT $GOase_{ox}$	UV-Vis [a]	5.7	7.0	10
	K (NCS^-)	<6.0	7.0	10
	K (N_3^-)	<6.0	7.2	10
	k_f (NCS^-)	<6.0	~7.0	10
WT $GOase_{semi}$	UV-Vis [a]	5.6	8.0	10
	UV-Vis		7.9	4
	Redox		8.0 [b]	4
Tyr495Phe $GOase_{ox}$	UV-Vis [a]	5.7		10
	K (NCS^-)	<6.0		10
Trp290His $GOase_{semi}$	UV-Vis		6.9	5

[a] No buffer present

[b] Average of values 7.8-8.1 for four different oxidants.

Substitution Reactions at the Substrate Binding Site

Reactions of $GOase_{ox}$ and $GOase_{semi}$ have been studied, where initially H_2O occupies the exogenous binding site. Incoming ligands $Y^- = NCS^-$, N_3^- were chosen to mimic RCH_2OH substrate binding. Two other ligands $Y^- = CH_3CO_2^-$ and $H_2PO_4^-$ were of interest because of their role as buffers, and because acetate is coordinated in one of the crystal structures. However the studies described here with acetate were at pH's out of the buffer range. The equilibration reactions for $GOase_{ox}$ or $GOase_{semi}$, (4),

$$GOase\ H_2O/OH^- + Y^- \rightleftharpoons \quad GOase\ Y^- + H_2O/OH^- \qquad (4)$$

define K, as well as forward and back rate constants k_f and k_b respectively. Values of K (25°C) were determined by titration of Y^- from a microsyringe into solutions of $GOase_{ox}$ or $Goase_{semi}$, and monitoring the UV-Vis changes at pH 7.0, I = 0.100M (NaCl). For NCS^- and N_3^- 10mM phosphate buffer was used. For acetate which is much less strongly binding 10mM lutidine buffer was used. On replacing Cl^- by ClO_4^- no change in UV-Vis spectrum was observed. X-ray crystal structure determinations indicate that N_3^- and $CH_3CO_2^-$ occupy the exogenous position.[6,7] The rate constants k_f and k_b for NCS^- and N_3^- were determined by stopped-flow method. Information obtained is listed in Table 2.

Table 2: Summary of formation equilibrium constants (K) and corresponding rate constants (k_f and k_b) for the complexing of anions at the substrate binding sites of different wild-type (WT) galactose oxidase (GOase) enzyme states at pH 7.0 (10mM buffer PO_4), I = 0.100M NaCl.

Reaction	K (dm^3mol^{-1})	k_f ($dm^3mol^{-1}s^{-1}$)	k_b s^{-1}
WT $GOase_{ox}$ + NCS^-	500	1.1×10^4	22.5
WT $GOase_{ox}$ + N_3^-	1.92×10^4	5.0×10^5	24.6
WT $GOase_{ox}$ + $CH_3CO_2^-$	104		
WT $GOase_{ox}$ + $H_2PO_4^-$ [a]	46		
WT $GOase_{semi}$ + NCS^-	270		
Tyr495Phe $GOase_{ox}$ + NCS^-	1.0×10^5		

[a] $H_2PO_4^-$ and HPO_4^- in equilibrium (pK_a 7.0)

Catalytic Process

Enzyme kinetic studies on the reaction of $GOase_{ox}$ (2-8μM) with D-galactose and D-raffinose (2-100mmol dm^{-3}) in the presence of O_2 (0.26mmol dm^{-3}) were carried out at pH 7.0 (10mmol dm^{-3} phosphate).[4,9] In 10mM phosphate, using K = 46dm^3 mol^{-1} from Table 2, there is 32% binding of phosphate, and this fraction of binding sites may not be immediately available to the substrate. Values of K_M and k_{cat} are 0.15M and 5500s^{-1} for D-galactose, and 0.07M and 4200s^{-1} for D-raffinose.[9] The reciprocal of K_M indicates the extent of binding, K_{bind} = 6.7 (D-galactose) and 14.3 (D-raffinose), of substrate for H_2O at the exogenous site. The small values reflect stereospecific restrictions on the reactions of a wide range of substrates which can be utilised in

such studies.[11] Since the enzyme is extra cellular high K_{bind} values are not required. Values of k_{cat} corresponding to the redox process $RCH_2OH \rightarrow RCHO$. The rate constant k_2 in (3) is 1.01 x 10^7 dm^3 $mol^{-1}s^{-1}$.

Effect of Coordinated NCS^- and N_3^- on pK_{2a}

From K (25°C) values for the complexing of NCS^- and N_3^- to wild-type $GOase_{ox}$, Table 2, it was calculated that 1.10mole dm^{-3} (NCS^-) and 0.0048mol dm^{-3} (N_3^-) give >98% anion binding at the exogenous site at pH 7.0, I = 0.100M (NaCl). $GOase_{ox}$ solutions were prepared at pH 5.76 and at pH 8.05, and the anion concentrations adjusted to the levels indicated. The pH was then varied over the range 5.76 - 8.05. No variations in UV-Vis absorbance were noted. Furthermore the H^+ is not able to access or leave the Tyr-495 site once the anions are in place. It is concluded that the same very likely holds when other groups such as the RCH_2OH^- substrate molecules are present.

Mechanism

Prior or subsequent loss of a proton from substrate $RCH_2OH \rightarrow RCH_2O^-$ at the time of coordination is we believe an essential part of the mechanism. The crucial step is then H-atom transfer from the coordinated RCH_2O^- to the radical at Tyr-272, which is accompanied by electron transfer to the Cu^{II}. Rate constants k_{cat}/s^{-1} for this process, D-galactose (5500) and D-raffinose (4200), are very similar. Release of RCHO follows, where aldehydes are known to be poor coordinating groups. From stopped-flow kinetic studies on the D-galactose and D-raffinose reactions a 2-fold increase in rate constants $k_1/M^{-1}s^{-1}$ is observed over the pH range 5-9. The pK_a corresponding to these changes is close to 7.0 (Table 1). The variations observed are dependent on whether the axial Tyr-495 is protonated or not. The relatively small changes in k_1 ascribed here to protonation of the axial Tyr-495 have led to the assumption that Tyr-495 does not dissociate. The Tyr-495Phe variant on the other hand exhibits no enzyme activity.

Acknowledgements

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