Kinetic Studies on the Redox Interconversion of $GOase_{semi}$ and $GOase_{ox}$ Forms of Galactose Oxidase with Inorganic Complexes as Redox Partners

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Redox interconversions between the $GOase_{semi}$ (Cu^{II}, Tyr) and tyrosyl radical containing $GOase_{ox}$ (Cu^{II}, $Tyr^{\bullet}$) oxidation states of the Cu-containing enzyme galactose oxidase (GOase) from *Fusarium* NRRL 2903 have been studied. The inorganic complexes $[Fe(CN)_6]^{3-}$ (410 mV), $[Co(phen)_3]^{3+}$ (370 mV), $[W(CN)_8]^{3-}$ (530 mV), and $[Co(dipic)_2]^{-}$ (362 mV) ($E^{\circ\prime}$ 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 $GOase_{ox}$. On oxidation of $GOase_{semi}$ a radical is generated at the coordinated phenolate of Tyr-272 to give $GOase_{ox}$. The one-electron reduction potential $E^{\circ\prime}$ (25 °C) for the $GOase_{ox}/GOase_{semi}$ couple varies with pH and is 400 mV vs NHE at pH 7.5, the smallest value so far observed for a tyrosyl radical. 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^{-1} s^{-1} of 0.16×10^3 (pH ∼ 9.5) increases to 4.3×10^3 (pH ∼ 5.5), while for $[Co(phen)_3]^{3+}$ a value of 4.9×10^3 (pH ∼ 9.5) decreases to 0.04×10^3 (pH ∼ 5.5), $I = 0.100$ M (NaCl). From the kinetics a single $GOase_{semi}$ acid dissociation process, pK_a = 8.0 (average), has been confirmed by UV−vis spectrophotometric studies (7.9). The corresponding value for $GOase_{ox}$ is 6.7. No comparable kinetic or spectrophotometric pH dependences are observed with the Tyr495Phe variant, indicating the axial Tyr-495 as the site of protonation. Neutral CH_3CO_2H and HN_3 species bind at the substrate binding site of $GOase_{semi}$, thus mimicking the behavior of primary alcohols RCH_2OH, the natural substrate of GOase. On coordination, loss of a proton occurs, and inhibition of the oxidation with $[Fe(CN)_6]^{3-}$ is observed.

Introduction

Galactose oxide (GOase; EC 1.1.3.9), in the present case from the Canadian wood-rot fungus *Fusarium* NRRL 2903 (previously referred to as *Polyporus circinatus* and *Dactylium dendroides*),[1] is a single type 2 Cu-containing enzyme (M_r 68 kDa; 639 amino acids).[2−4] In its active form GOase is now known to contain a coordinated tyrosyl free radical,[5−7] and three oxidation states can be defined as in eq 1. X-ray structures

$$\underset{GOase_{ox}}{Cu^{2+}-Tyr^{\bullet}} \underset{-e^-}{\overset{+e^-}{\rightleftharpoons}} \underset{GOase_{semi}}{Cu^{2+}-Tyr} \underset{-e^-}{\overset{+e^-}{\rightleftharpoons}} \underset{GOase_{red}}{Cu^{+}-Tyr} \qquad (1)$$

have been reported on crystals obtained from "native" $GOase_{ox}/GOase_{semi}$ enzyme mixes, with H_2O (resolution 1.9 Å),[8] acetate (1.7 Å),[8,9] and azide (1.9 Å)[10] coordinated at the substrate binding site. The Cu^{II} has a square-pyramidal geometry and is coordinated by Tyr-272, Tyr-495 (axial ligand), His-496 and His-581 in addition to the exogenous H_2O (or acetate/azide) at position X, Figure 1. Bond lengths can be regarded as normal for Cu^{II}, except for *a* and *b*, which are long, as indicated in Figure 1. The same structure is obtained for crystals grown in solutions of the oxidant $[Fe(CN)_6]^{3-}$. Exafs data have indicated that there is no significant change in the active site structure for the $GOase_{semi}$ and $GOase_{ox}$ forms.[11] In the catalytic cycle the two-equivalent reduction of $GOase_{ox}$ with RCH_2OH primary alcohol/substrates (eq 2) is followed by the reaction with O_2 (eq 3).[12] Accordingly GOase is a two-equivalent oxidase in

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

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

contrast to, e.g., the four-equivalent ascorbate oxidase, laccase, and cytochrome *c* oxidase, which convert O_2 to $2H_2O$.[4,13] Although the precise physiological function of GOase is not clear, it has been suggested that it may play some part in lignin degradation.[14] Autoredox processes result in substantial (∼95%) conversion of $GOase_{ox}$ to $GOase_{semi}$ within 3 h (25 °C) at pH 7.5 (and similar ∼5% conversion of $GOase_{semi}$ to $GOase_{ox}$), giving the so-called "native" mix.[15] This mix has been used

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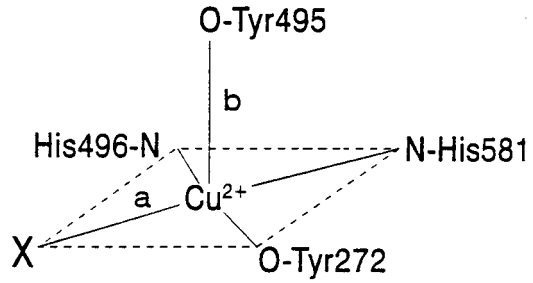

Ligand (X)	a/Å	b/Å	Ref
$CH_3CO_2^-$	2.30	2.70	8,9
H_2O	2.80	2.60	9
N_3^-	2.45	2.95	10

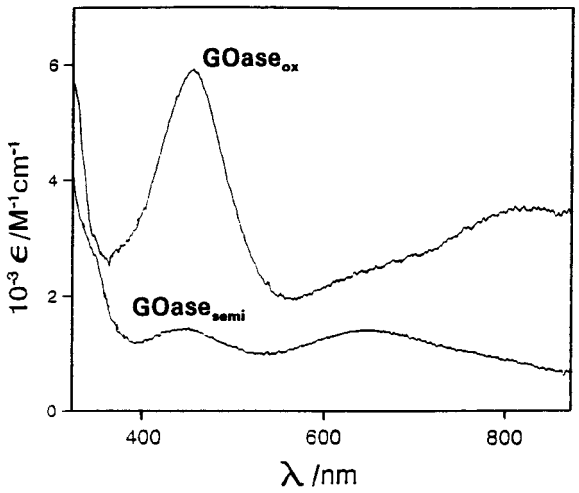

Figure 1. The Cu^{II} active site of GOase, crystal structure bond lengths *a* and *b* from refs 8−10, and UV−vis spectra at pH 7.5 (10 mM Hepes).

extensively in earlier studies,[16,17] prior to identification of the radical in 1988.[6]

In the present paper we study reactions with a range of inorganic redox partners, and the effect of pH on the interconversion of GOase$_{semi}$ and GOase$_{ox}$. The latter has a radical on the phenolate side chain of Tyr-272, which is covalently bonded at its ortho position to the cysteinyl S-atom of Cys-228, forming a unique thioether link.[8] The bond is overlaid by the indole ring of Trp-290, giving an unusually stable tyrosyl radical of low reduction potential ($E^{\circ\prime}$). Variations of $E^{\circ\prime}$ with pH are reported as a part of this work, and effects of pH on the kinetics have been assigned using the Tyr495Phe variant.

Experimental Section

Isolation of Enzyme. GOase was initially isolated from the native fungal source *Fusarium* (strain NRRL 2903).[18,19] Subsequently it was obtained from an *Aspergillus nidulans* (strain G191/pGOF101) expression system by a procedure already described.[20] The enzyme is strongly basic (pI = 12)[22] with a charge balance from Glu, Asp (negative) and Lys, Arg (positive) residues of ∼+9 for GOase$_{semi}$ at neutral pH. Final purification was performed by phosphocellulose column chromatography.[20] The same procedures were adopted for purifying the Tyr495Phe variant.[20,23] Yields of enzyme were ∼35 mg/L of culture, where 5 L was generally used per preparation.

Buffers. The following buffers were obtained from Sigma except as stated: 2-(*N*-morpholino)ethanesulfonic acid (Mes, pH 5.5−6.7, pK_a = 6.1); Na_2HPO_4 and NaH_2PO_4 (pH 5.5−8.2, pK_a = 7.00); 2,6-dimethylpyridine (lutidine, pH 6.0−8.0, pK_a = 6.75; Fluka); *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (Hepes, pH 6.8−8.2, pK_a = 7.5); and 2-(*N*-cyclohexylamino)ethanesulfonic acid (Ches, pH 8.6−10.0, pK_a = 9.3). All buffer solutions were prepared on the day of use or were stored over ∼1 day periods at 4 °C to avoid microbial growth. The buffers phosphate and Hepes have been shown to interact at the active site of the Trp290His GOase variant, with no similar effects of Mes, lutidine, or Ches.[25] No effects were observed with wild-type (WT) and Tyr495Phe GOase as used in these studies, except over long times >1 h with Hepes, when small changes were noted.

Enzyme Concentrations. These were determined for wild-type and Tyr495Phe GOase forms from the UV absorbance at 280 nm (ϵ = 1.05 × 10^5 M^{-1} cm^{-1}).[24] The buffers Mes, Hepes, phosphate, and Ches have negligible absorbance at 280 nm, and corrections were made for the absorbance of lutidine at <300 nm.

Preparation of Enzyme Solutions. Typically a 2 mL enzyme sample (3−5 mg/mL) was dialyzed against the appropriate buffer (10 mM) for ∼16 h at 4 °C. The dialysis solution was changed three times during this period. In order to oxidize or reduce native GOase, an excess of $[Fe(CN)_6]^{3-}$ or $[Fe(CN)_6]^{4-}$ was added, and it was removed by Amicon ultrafiltration immediately prior to use. To avoid contributions from autoredox processes during UV−vis studies, measurements were carried out with 2 equiv of $[Fe(CN)_6]^{3-}$ (to GOase$_{ox}$) or $[Fe(CN_6]^{4-}$ (to GOase$_{semi}$) added (5 μL aliquot), also immediately prior to use. No effect of $[Fe(CN)_6]^{3-}$ complexation on the spectrum of GOase$_{ox}$ was observed. UV−vis spectra of GOase$_{semi}$ and GOase$_{ox}$ at pH 7.5 are shown in Figure 1.

Inorganic Complexes. Potassium hexacyanoferrate(III), $K_3[Fe(CN)_6]$ (Sigma), and potassium hexacyanoferrate(II), $K_4[Fe(CN)_6]\cdot 3H_2O$ (BDH, Analar), were used as supplied. Samples of tris(1,10-phenanthroline)cobalt(III) as $[Co(phen)_3]Cl_3\cdot 7H_2O$ were prepared according to a procedure described,[26] with minor modifications. Characterization was according to the UV−vis absorbance spectrum, peaks λ/nm (ϵ/M^{-1} cm^{-1}) at 330 (4660) and 350 (3620). Solutions of tris(1,10-phenanthroline)cobalt(II) were obtained by adding a 5:1 ratio of 1,10-phenanthroline monohydrate to $CoCl_2\cdot 6H_2O$. Solutions prepared under anaerobic conditions were used immediately after preparation. The complex $Cs_3[W(CN)_8]\cdot 2H_2O$, peak positions 357 (1700), was obtained by a literature procedure.[27] Ammonium bis(pyridine-2,6-dicarboxylate)-cobalt(III), $NH_4[Co(dipic)_2]\cdot H_2O$, was prepared as described,[28] absorbance peak at 510 (630). In recent papers we have quoted the reduction potential ($E^{\circ\prime}$) of 747 mV for the $[Co(dipic)_2]^{-/2-}$ couple determined by Williams and Yandall.[29] In response to a referee comment we have now checked this value by electrochemical methods. In cyclic voltammetry experiments the oxidation step was not clearly defined. Using square-wave voltammetry on $[Co(dipic)_2]^-$ (7.4 mM) at pH 7.5

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(10 mM Tris), $I = 0.100$ M (NaCl), a reduction potential of 362(4) mV (22 °C) was determined. This value is in agreement with a value of 400 mV used in earlier papers from the Gray group.[28] It is moreover similar to the value for the $[Co(edta)]^{-/2-}$ couple of 380 mV (also CoO_4N_2 coordination). The smaller $E^{o\prime}$ does not affect the outcome of our studies, except that a >20-fold excess of $[Co(dipic)_2]^-$ is required for reactions to proceed to >90% completion.

Kinetic and Thermodynamic Studies. Protein solutions were dialyzed to the required pH, as described above. The protein is unstable at pH < 5.5, and an upper limit for pH in the range 9−10 was used. In most kinetic experiments the pH-jump method was employed, with the protein at low buffer concentration (10 mM), and the redox reagent made up in 100 mM buffer which was pH controlling. In this way the final pH (55 mM buffer) could be varied over 2 pH units, using a single protein solution and different solutions of redox reagent. Some check experiments were carried out with both the GOase and redox reagent made up at the same pH. The ionic strength was adjusted to I = 0.100 ± 0.001 M using NaCl, except for studies at $I = 0.40$ M (NaCl) or in the presence of acetate, $I = 0.40$ M (CH_3CO_2Na). Contributions of the enzyme (~10^{-5} M) to the ionic strength were assumed to be negligible. The temperature was 25.0 ± 0.1 °C. When phosphate was used, maximum (final) concentrations ~35 mM only were attainable due to the higher ionic strength contributions of the salts used. Dionex D-110 and Applied Photophysics stopped-flow instruments complete with kinetic software fitting packages were used. All rate constants indicated are an average from at least five stopped-flow traces. A Shimadzu 2100PC UV−vis spectrophotometer was employed for some $[Co(dipic)_2]^-$ runs, and for the spectrophotometric determination of the pK_a for $GOase_{semi}$.

Results

UV−Vis Determination of pK_a for $GOase_{semi}$. UV−vis spectra of $GOase_{semi}$ are dependent on pH, Figure 2. A color change to a more intense green-blue is observed with increasing pH. Absorbance changes vs pH at optimum wavelengths (450 and 635 nm) are shown in the inset to Figure 2. The acid dissociation constant K_a is defined in eq 4. For this equilibration eq 5 can be derived, which relates absorbance readings for equilibrated solutions (A_{obs}) to those for $GOase_{semi}$ (A_0) and the protonated form $GOase_{semi}H^+$ (A_H).

$$GOase_{semi}H^+ \overset{K_a}{\rightleftharpoons} GOase_{semi} + H^+ \quad (4)$$

$$\frac{1}{(A_{obs} - A_H)} = \frac{[H^+]}{K_a(A_0 - A_H)} + \frac{1}{(A_0 - A_H)} \quad (5)$$

The ratio intercept/slope ratio from a plot of $(A_{obs} - A_H)^{-1}$ vs $[H^+]$ gives K_a. The pK_a values obtained average 7.9(2). The corresponding value determined for $GOase_{ox}$ is 6.7(2).[15]

UV−Vis Studies on the Tyr495Phe Variant. The $GOase_{semi}$ and $GOase_{ox}$ forms of the Tyr495Phe variant have spectra that are different from those of the wild type protein. The differences are particularly marked for $GOase_{ox}$, for which there is no 810 nm peak, and the 450 nm peak is less intense. Both these observations are in keeping with Whittaker's assignments for WT GOase.[3] The effect of changing the pH from 5.0 to 9.0 on the UV−vis spectrum of Tyr495Phe $GOase_{semi}$ at the 610 nm peak is negligible (±4%) compared to the effect in the case of WT $GOase_{semi}$. With Tyr495Phe $GOase_{ox}$ the changes are also small, and again no protonation is evident.[15]

Kinetic Studies on the Oxidation of $GOase_{semi}$. Four reactions were studied with $[Fe(CN)_6]^{3-}$ (410 mV), $[Co(phen)_3]^{3+}$ (370 mV), $[W(CN)_8]^{3-}$ (530 mV), and $[Co(dipic)_2]^-$ (362 mV) as one-equivalent oxidants. The reactions required stopped-flow monitoring, with the slower $[Co(dipic)_2]^-$ reaction studied in part by conventional UV−vis spectrophotometry. First-order rate constants k_{obs} were determined with the oxidant

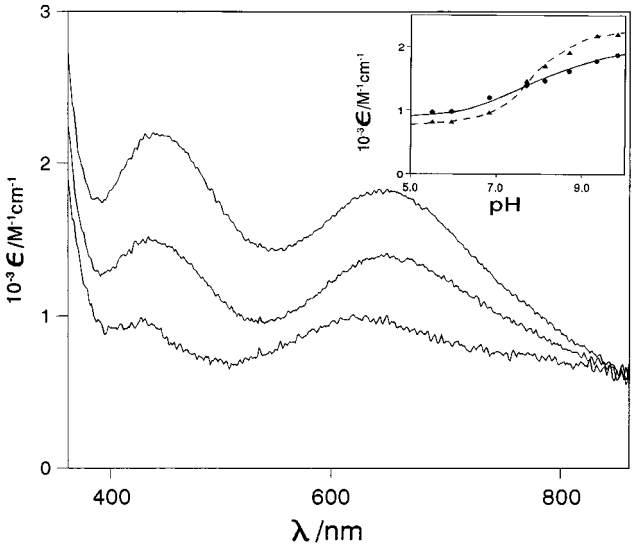

Figure 2. The effect of pH on the UV−vis spectrum of $GOase_{semi}$ (25 °C): upper spectrum at pH 9.34 (Ches), middle at pH 7.70 (Hepes), and lower at pH 5.95 (Mes), $I = 0.100$ M (NaCl). The inset shows pH vs ϵ at 450 nm (−▲−) and 635 nm (−●−).

in ≥10-fold excess. At selected pH's (3−4 for each reaction) a first-order dependence of k_{obs} on oxidant concentration was demonstrated, allowing second-order rate constants k_{Fe}, k_{Co}, k_W, and k_{CoD} respectively to be determined. The dependence of such rate constants on pH (listings in Supporting Information) are illustrated in Figures 3 and 4. To explain the variations observed the reaction sequence 6−8 is proposed. The expression 9 can

$$GOase_{semi}H^+ \overset{K_a}{\rightleftharpoons} GOase_{ox} + H^+ \quad (6)$$

$$GOase_{semi}H^+ + \text{oxidant} \xrightarrow{k_H} GOase_{ox}H^+ + \text{reduced} \quad (7)$$

$$GOase_{semi} + \text{oxidant} \xrightarrow{k_0} GOase_{ox} + \text{reduced} \quad (8)$$

be derived for the rate constant k_{Fe} with $[Fe(CN)_6]^{3-}$ as oxidant, which gives a good fit using an unweighted nonlinear least-squares program. With $[Co(dipic)_2]^-$, the oxidant of smallest

$$k_{Fe} = \frac{k_H[H^+] + k_0K_a}{K_a + [H^+]} \quad (9)$$

charge, both k_H and k_0 clearly contribute, Figure 4, and both terms are therefore retained for the other three studies, although one term in each case is sufficiently small to question its contribution. The results are summarized in Table 1. The average pK_a of 8.0(2) is in good agreement with the value 7.9-(2) determined by UV−vis spectrophotometry.

With two of the oxidants, $[Fe(CN)_6]^{3-}$ and $[Co(phen)_3]^{3+}$, rate constants were also determined with the Tyr495Phe variant of $GOase_{semi}$ (Supporting Information), and these results are also indicated in Figure 3. It is concluded that there is no pH dependence in the reaction with $[Fe(CN)_6]^{3-}$, and with $[Co(phen)_3]^{3+}$ the slight upward trend does not relate to a specific change at the active site.

Kinetic Studies on the Reduction of $GOase_{ox}$. It is possible to study the reductions of $GOase_{ox}$ with $[Fe(CN)_6]^{4-}$ and $[Co(phen)_3]^{2+}$ (present in >10-fold excess). First-order rate constants k_{obs} again give linear dependencies on the concentration of reductant, and second-order rate constants k_{-Fe} and k_{-Co}

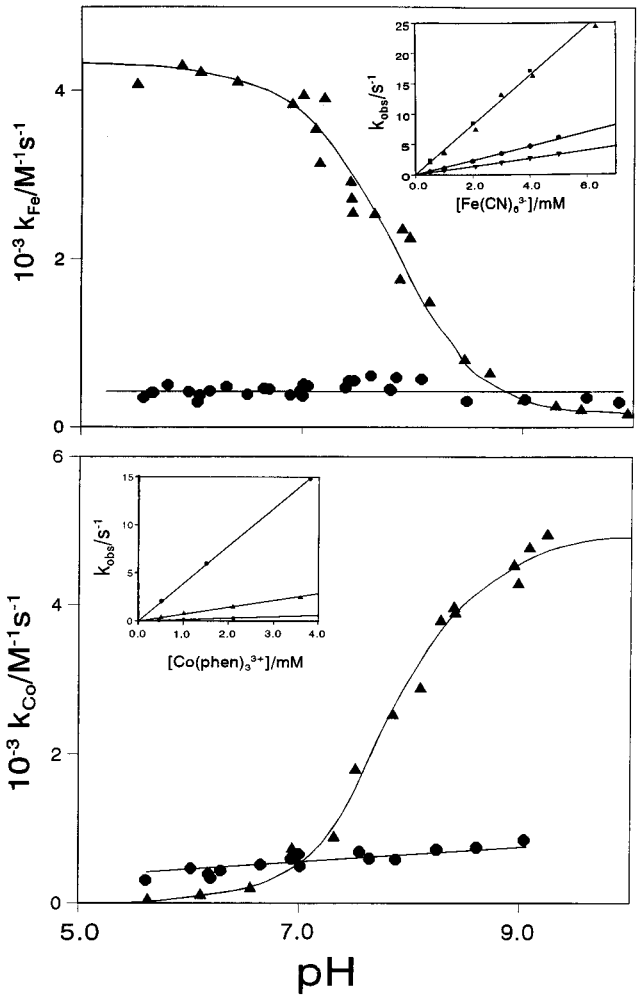

Figure 3. (A, top) The variation of second-order rate constants k_{Fe} (25 °C) with pH for the $[Fe(CN)_6]^{3-}$ oxidation of GOase$_{semi}$ (▲) and Tyr495Phe GOase$_{semi}$ (●), $I = 0.100$ M (NaCl), and (B, bottom) the variation of second-order rate constants k_{Co} (25 °C) with pH for the $[Co(phen)_3]^{3+}$ oxidation of GOase$_{semi}$ (▲) and Tyr495Phe GOase$_{semi}$ (●), $I = 0.100$ M (NaCl). The insets show respectively the dependence of first-order rate constants k_{obs} for the oxidation of GOase$_{semi}$ on $[Fe(CN)_6^{3-}]$ at pH 5.50 (■), 6.99 (▲), 8.02 (●), and 8.60 (▼), and on $[Co(phen)_3^{3+}]$ at pH 6.10 (■), 6.94 (▲), and 8.40 (●).

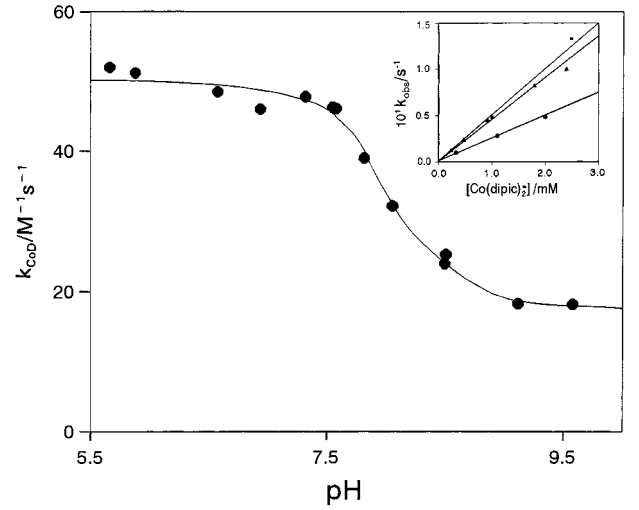

Figure 4. The variation of second-order rate constants k_{CoD} (25 °C) with pH for the $[Co(dipic)_2]^-$ oxidation of GOase$_{semi}$, $I = 0.100$ M (NaCl). The inset shows the dependence of first-order rate constants k_{obs} on $[Co(dipic)_2^-]$ at pH 5.66 (■), 6.94 (▲), and 8.50 (●).

were determined. The variations with pH are illustrated in Figure 5 (listing in Supporting Information). Two effects of pH appear to contribute. One of these is determined by the GOase$_{ox}$ pK_a of 6.7,[15] and the second, effective at lower pH, is due to a further protonation which we are not at present able to assign. The $[Fe(CN)_6]^{4-}$ reduction of Tyr495Phe GOase$_{ox}$ gives no variation in rate constant over the range pH 7.5−9.5, but again at low pH the unassigned effect appears to contribute. Rate constants for the corresponding reaction with $[Co(phen)_3]^{2+}$, inset Figure 5, show a 29% decrease with increasing pH from 5.9 to 9.1, and again these do not appear to relate to a single protonation step (listing in Supporting Information).

Table 1. Summary of Rate Constants (25 °C) k_H (Low pH) and k_0 (High pH) and GOase Acid Dissociation Constants K_a for WT GOase and a Comparison with pK_a Values from Spectrophotometry[a]

reaction	k_H/M^{-1} s^{-1}	k_0/M^{-1} s^{-1}	pK_a
GOase$_{semi}$ + $[Fe(CN)_6]^{3-}$	$4.3(1) \times 10^3$	$0.16(12) \times 10^3$	7.8(1)
GOase$_{semi}$ + $[Co(phen)_3]^{3+}$	$0.04(10) \times 10^3$	$4.9(1) \times 10^3$	7.9(1)
GOase$_{semi}$ + $[W(CN)_8]^{3-}$	$5.5(1) \times 10^4$	$0.38(1) \times 10^4$	8.0(1)
GOase$_{semi}$ + $[Co(dipic)_2]^-$	51(1)	16(2)	8.1(1)
spectrophotometry GOase$_{semi}$			7.9(2)
spectrophotometry GOase$_{ox}$			6.7(2)[b]

[a] $I = 0.100$ M (NaCl) unless otherwise indicated. [b] See ref 15.

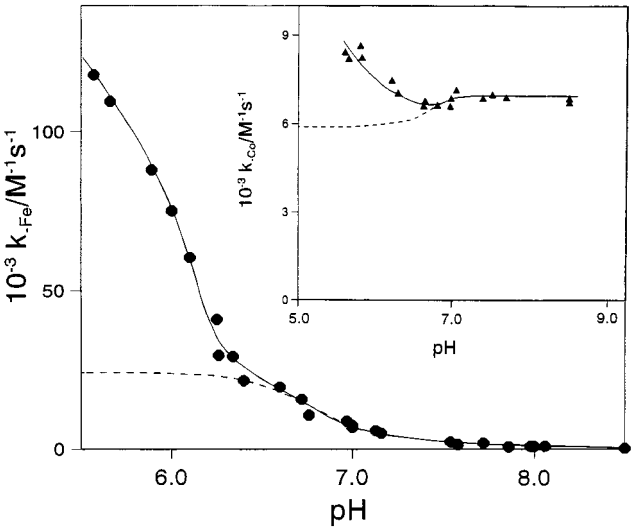

Figure 5. The variation of second-order rate constants k_{-Fe} (25 °C) with pH for the $[Fe(CN)_6]^{4-}$ reduction of GOase$_{ox}$. The inset shows the corresponding variation of the $[Co(phen)_3]^{2+}$ reduction of GOase$_{ox}$, I = 0.100 M (NaCl). The broken lines are an extrapolation indicating the influence of the spectrophotometric pK_a of ∼6.7.

Reduction Potential of the GOase$_{ox}$/GOase$_{semi}$ Couple. It is possible to determine $E^{o\prime}$ (vs NHE) from rate constants for redox studies involving the $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ ($E^{o\prime} = 410$ mV) and $[Co(phen)_3]^{3+}/[Co(phen)_3]^{2+}$ ($E^{o\prime} = 370$ mV) couples. Thus eqs 10 and 11 apply, and $\Delta E^{o\prime}$ can be defined as in eq 12. Values of $E^{o\prime}$ for the GOase$_{ox}$/GOase$_{semi}$ couple are in good

$$\text{GOase}_{semi} + [Fe(CN)_6]^{3-} \underset{k_{-Fe}}{\overset{k_{Fe}}{\rightleftharpoons}} \text{GOase}_{ox} + [Fe(CN)_6]^{4-} \quad (10)$$

$$\text{GOase}_{semi} + [Co(phen)_3]^{3+} \underset{k_{-Co}}{\overset{k_{Co}}{\rightleftharpoons}} \text{GOase}_{ox} + [Co(phen)_3]^{2+} \quad (11)$$

$$\frac{\Delta E^{o\prime}}{0.059} = \log_{10}(k_{Fe}/k_{-Fe}) \quad \text{or} \quad \log_{10}(k_{Co}/k_{-Co}) \quad (12)$$

agreement and are dependent on pH as shown in Figure 6. Corresponding $E^{o\prime}$ values for the Tyr495Phe variant have been obtained and give no dependence on pH in the range 6−9, Figure 6.

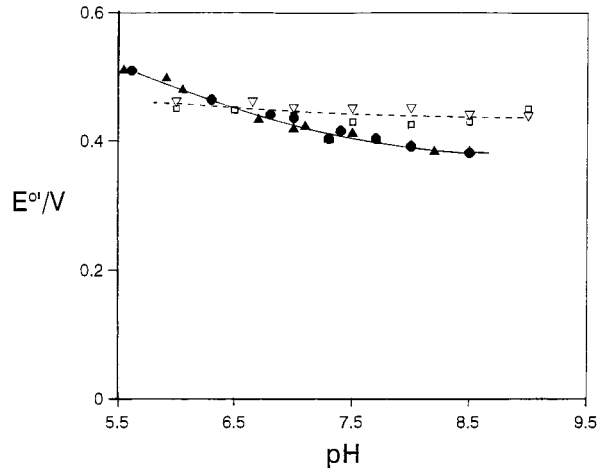

Figure 6. The variation of reduction potential $E^{\circ\prime}$ vs NHE (25 °C) for the $GOase_{ox}/GOase_{semi}$ couple with pH for wild-type enzyme (—) and the Tyr495Phe variant (− − −), $I = 0.100$ M (NaCl). The $E^{\circ\prime}$ values were obtained from rate constants for the $[Co(phen)_3]^{3+/2+}$ reactions (●, wild-type; ▽ Tyr495Phe) and $[Fe(CN)_6]^{3-/4-}$ (▲, wild-type; □, Tyr495Phe).

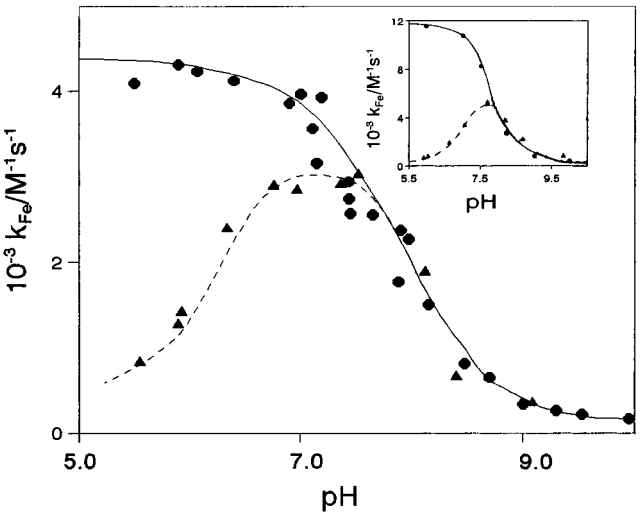

Figure 7. Variation of second-order rate constants k_{Fe} (25 °C) with pH for the $[Fe(CN)_6]^{3-}$ oxidation of $GOase_{semi}$ (●) and the effect of (0.06−0.09 M) acetate (−▲−), $I = 0.100$ M. The inset shows the same study carried out on $GOase_{semi}$ in the presence of 0.37 M acetate, $I = 0.400$ M (NaO_2CCH_3).

Effect of Acetate and Azide on the $[Fe(CN)_6]^{3-}$ Oxidation of $GOase_{semi}$. Similar behavior is observed in both cases, acetate (~80 mM) and azide (1.5−2.0 mM), with little or no effect at high pH, and approaching a switch-off in reactivity at low pH, Figures 7 and 8. Rate constants were also determined at the higher ionic strength of 0.40 M (with acetate to 0.37 M) to give more extensive complexing at the substrate binding site, inset to Figure 7. With $CH_3CO_2^-$ coordinated instead of H_2O, reaction with $[Fe(CN)_6]^{3-}$ is less favorable. This effect is dominant over any advantage resulting from protonation at Tyr-495. No effect of acetate (0.08 M) is observed with $[Co(phen)_3]^{3+}$ as oxidant for $GOase_{semi}$ at pH 5.8, 7.2, and 8.8. Acid dissociation pK_a values for CH_3CO_2H (4.55)[30] and HN_3 (4.44)[31] suggest that protonation of coordinated acetate or azide is unlikely.

Discussion

Rate constants for the oxidation of $GOase_{semi}$ with $[Fe(CN)_6]^{3-}$, $[Co(phen)_3]^{3+}$, $[W(CN)_8]^{3-}$, and $[Co(dipic)_2]^-$ as oxidants show

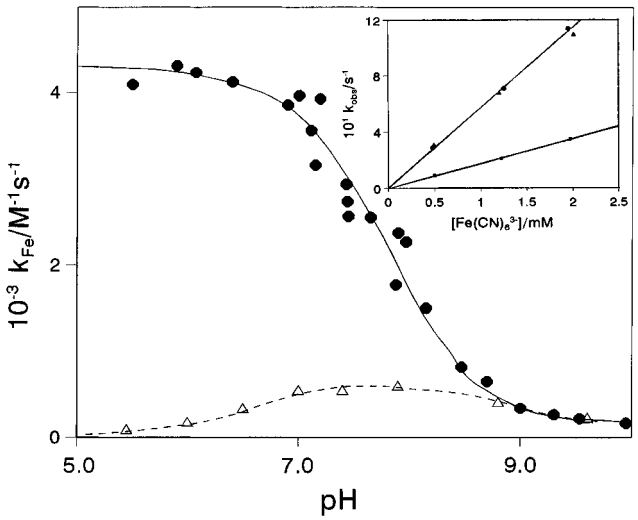

Figure 8. Variation of second-order rate constants k_{Fe} (25 °C) with pH for the $[Fe(CN)_6]^{3-}$ oxidation of $GOase_{semi}$ (●) and with (1.50−5.0) × 10^{-3} M azide added (−△−), $I = 0.100$ M. The inset shows the dependence of the first-order rate constant k_{obs} on $[Fe(CN)_6]^{3-}$ in the presence of azide at pH 5.99 (■), 6.98 (▲), 7.97 (●).

dependences on pH, from which a single proton pK_a of 8.0 (av) is obtained, indicating a common process. The same pK_a (7.9) is obtained from UV−vis spectrophotometric studies on $GOase_{semi}$. No pH effects are observed with the Tyr495Phe variant, and protonation is therefore assigned to the axial Tyr-495.

Crystallographic distances reported for the axial Cu^{II}−O (Tyr-495) bond of 2.60 Å (H_2O form),[8] 2.70 Å (acetate),[8,9] and 2.95 Å (azide)[10] indicate long bonds appropriate to the square pyramidal structure. It is not clear whether in these structures the Tyr-495 is protonated. The rate constants obtained with low charged $[Co(dipic)_2]^-$ as oxidant, Figure 4, indicate contributions to the reaction from both k_H (protonated $GOase_{semi}$) and k_0 (unprotonated $GOase_{semi}$) with a k_H/k_0 ratio of 3.2, Table 1. With the two 3 − oxidants the corresponding ratios are $[Fe(CN)_6]^{3-}$ (27) and $[W(CN)_8]^{3-}$ (15), but with $[Co(phen)_3]^{3+}$ a ratio k_0/k_H of 123 is obtained, and k_H makes little or no contribution. The variations in rate constants with pH indicate an extreme sensitivity to oxidant charge. With anionic oxidants the $GOase_{semi}$ benefits from protonation at Tyr-495, and with the cationic oxidant the opposite holds. The high sensitivity of reactions to protonation at the axial Tyr-495 is consistent with its close proximity cis to the substrate binding site.

Rate constants have also been determined with pH for the $[Fe(CN)_6]^{4-}$ and $[Co(phen)_3]^{2+}$ reductions of $GOase_{ox}$, when the Tyr-495 pK_a is 6.7.[15] Here the sensitivity of reactions is not as extreme, and an additional pH effect appears to contribute at pH <6.5, Figure 5. From the ratio of rate constants for the redox reactions with $[Fe(CN)_6]^{3-/4-}$ and $[Co(phen)_3]^{3+/2+}$, the reduction potential ($E^{\circ\prime}$) for the $GOase_{ox}/GOase_{semi}$ couple has been determined, Figure 6. The redox change involves formation of the radical at Tyr-272. The $E^{\circ\prime}$ varies from 0.380 V (pH 8.5), to 0.500 V (pH 5.5), and at pH 7.5 it is 0.400 V. There is good agreement with previous $E^{\circ\prime}$ values determined by thin layer electrochemistry,[32] but in the latter study the reaction was incorrectly assigned as a Cu^{II}/Cu^{I} change. The 60 mV/pH unit variation[32] arises from the Tyr-495 pK_a values for $GOase_{semi}$ (8.0) and $GOase_{ox}$ (6.7). In the case of the Tyr495Phe variant the $E^{\circ\prime}$ of 0.425 V is independent of pH. Values for $E^{\circ\prime}$ with pH from 0.380 to 0.500 V are small bearing in mind the magnitude of $E^{\circ\prime}$ for other tyrosyl radical systems. Thus reduction potentials for the $Tyr^{\bullet}$/TyrH couple in a

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Table 2. A Summary of Self-Exchange Rate Constants $k_{11}/M^{-1}\ s^{-1}$ (25 °C) and Reduction Potential $E^{\circ\prime}$ (V) for Redox Couples Used in These Studies[a]

reagent	$E^{\circ\prime}$ (V)	$k_{11}/M^{-1}\ s^{-1}$	$k_{12}/M^{-1}\ s^{-1}$	ref
$[Fe(CN)_6]^{3-/4-}$	0.410	2.6×10^4	2.0×10^3	36, 37
$[Co(phen)_3]^{3+/2+}$	0.370	12	3.5×10^3	26
$[W(CN)_8]^{3-/4-}$	0.540	4×10^4	3.0×10^3	27
$[Co(dipic)_2]^{-/2-}$	0.362[b]	1×10^{-5}	40	this work

[a] The rate constants k_{12} were those determined at pH 7.5, see Figures 3−6. [b] In ref 28 a value 400 mV is used.

nonprotein environment are substantially larger (0.78−0.94 V),[33] and in the case of ribonucleotide reductase the most recent estimate is ∼1.0 V.[34] The value for GOase is the smallest so far obtained for a tyrosyl radical and is accounted for by the coordination of the phenolate of Tyr-272, as well as additional structural features including the bonding of the S of Cys-228 at the ortho position of the phenolate, and the overlaying of this bond by the indole ring of Trp-290. Variant GOase forms in which Cys-228 is removed,[35] and Trp-290 replaced,[25] exhibit decreased reactivity which can be explained by larger $E^{\circ\prime}$ values. Electron self-exchange (ese) rate constants (k_{11}) and reduction potentials ($E^{\circ\prime}$) for the inorganic couples used in these studies are listed in Table 2.[26−28,36,37] The $[Co(dipic)_2]^-$ reaction is slow because of the low driving force and small k_{11}. Whether a single ese rate constant (k_{22}) can be obtained for galactose oxidase has been examined briefly. Rate constants (k_{12}) at pH 7.5 are listed in Table 2. Using the Marcus equation (13),[38,39] ese rate constants (k_{22}) for the $GOase_{ox}/GOase_{semi}$ exchange were calculated, where K_{12} is the thermodynamic driving force (Table 2), and the constant f is assumed to be unity for reactions of small driving force as in the present case. With the cyano

$$k_{12} = (k_{11}k_{22}K_{12}f)^{1/2} \qquad (13)$$

complexes as oxidants $k_{22}/M^{-1}\ s^{-1}$ values are $\sim 10^2$, but with $[Co(phen)_3]^{3+}$ and $[Co(dipic)_2]^-$ these are $\sim 10^6$ and $\sim 10^8$, indicating the need for other factors to be taken into account.[39] From the structure coordinates[8] the closest possible approach of the two exchanging phenolate Tyr-272 O atoms is similar to that of the two Cu atoms and is ∼16 Å.

X-ray structures have confirmed that acetate and azide bind in place of H_2O at the substrate binding site.[9,10] The bond distance applying, Figure 1, at the substrate binding position $Cu^{II}-O$ (H_2O), 2.80 Å[8] is considerably shortened to 2.30 Å (acetate),[8,9] and is 2.45 Å for the $Cu^{II}-N$ (azide).[10] The crystals with acetate incorporated were grown at pH ∼4.5,[9] and those with azide by soaking the latter in azide at pH 7.0.[10] Rate constants obtained with $[Fe(CN)_6]^{3-}$ as oxidant for $GOase_{semi}$ in the presence of ∼0.080 M acetate at pH >7.5 are close to those obtained in the absence of acetate, suggesting that anionic $CH_3CO_2^-$ has difficulty in binding to the Cu. A similar situation applies also with azide ($\sim 2.0 \times 10^{-3}$ M), for which more detailed studies have been carried out.[40] At the lower pH's CH_3CO_2H and HN_3 (by analogy with substrate RCH_2OH) are able to access the active site and coordinate more extensively in place of H_2O. However, on coordination, acid dissociation to $CH_3CO_2^-$ and N_3^- occurs, and the charge (and size), cf. H_2O, are not as conducive to reaction with $[Fe(CN)_6]^{3-}$. The inhibitory effect and eventual close to switch-off behavior observed with $[Fe(CN)_6]^{3-}$ at low pH is surprising in view of the essentially nil effect observed with $[Co(phen)_3]^{3+}$. As a possible explanation, inner-sphere binding of $[Fe(CN)_6]^{3-}$ to the Cu has been considered. However, contrary to earlier reports,[41] we find no UV−vis spectrophotometric evidence for interaction of $[Fe(CN)_6]^{3-}$ (2.4 mM) with $GOase_{ox}$ (8 μM). Moreover, in crystallographic studies carried out on crystals of GOase soaked in 100 mM $[Fe(CN)_6]^{3-}$,[8] no change in the active site structure was observed.

To summarize, studies on the redox interconversion of $GOase_{semi}$ and $GOase_{ox}$ with inorganic complexes are able to target the formation and decay of the tyrosyl radical at Tyr-272. The $E^{\circ\prime}$ for the one-equivalent $GOase_{ox}/GOase_{semi}$ couple of 400 mV (pH 7.5) is small for a process involving tyrosyl radical formation and is independent of the inorganic redox couple used. Rate constants for the oxidation of $GOase_{semi}$ are sensitive to the charge on the oxidant as well as pH, the latter stemming from the protonation of Tyr-495 (pK_a 7.9). The smaller pK_a in the case of $GOase_{ox}$ (pK_a 6.7)[15] is in accordance with the higher positive charge of the active site. With $[Fe(CN)_6]^{4-}$ and $[Co(phen)_3]^{2+}$ as reductants for $GOase_{ox}$, protonation effects are also observed. With negatively charged acetate or azide at the H_2O site of $GOase_{semi}$, the reactivity with $[Fe(CN)_6]^{3-}$ decreases.

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Supporting Information Available: Tables listing rate constants (10 pages). Ordering information is given on any current masthead page.

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