Thermodynamic, Kinetic and pH Studies on the Reactions of NCS^-, N_3^-, and $CH_3CO_2^-$ with *Fusarium* Galactose Oxidase

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Thermodynamic and kinetic studies on the $X^- = NCS^-$, N_3^-, and $CH_3CO_2^-$ replacement of H_2O/OH^- at the Cu^{II} exogenous site of the tyrosyl-radical-containing enzyme galactose oxidase ($GOase_{ox}$) from *Fusarium* (NRR 2903), have been studied by methods involving UV−vis spectrophotometry (25 °C), pH range 5.5−8.7, $I = 0.100$ M (NaCl). In the case of N_3^- and $CH_3CO_2^-$ previous X-ray structures have confirmed coordination at the exogenous H_2O/OH^- site. From the effect of pH on the UV−vis spectrum of $GOase_{ox}$ under buffer-free conditions, acid dissociation constants of 5.7 (pK_{1a}; coordinated H_2O) and 7.0 (pK_{2a}; H^+Tyr-495) have been determined. At pH 7.0 formation constants K(25 °C)/M^{-1} are NCS^- (480), N_3^- (1.98×10^4), and $CH_3CO_2^-$ (104), and from the variations in K with pH the same two pK_a values are seen to apply. No pK_{1a} is observed when X^- is coordinated. From equilibration stopped-flow studies rate constants at pH 7.0 for the formation reaction k_f(25 °C)/M^{-1} s^{-1} are NCS^- (1.13×10^4) and N_3^- (5.2×10^5). Both K and k_f decrease with increasing pH, consistent with the electrostatic effect of replacing H_2O by OH^-. In the case of the $GOase_{ox}$ Tyr495Phe variant pK_{1a} is again 5.7, but no pK_{2a} is observed, confirming the latter as acid dissociation of protonated Tyr-495. At pH 7.0, K for the reaction of four-coordinate $GOase_{ox}$ Tyr495Phe with NCS^- ($1.02 \times 10^5 M^{-1}$) is more favorable than the value for $GOase_{ox}$. Effects of H^+Tyr-495 deprotonation on K are smaller than those for the H_2O/OH^- change. The pK_{1a} for $GOase_{semi}$ is very similar (5.6) to that for $GOase_{ox}$ (both at Cu^{II}), but pK_{2a} is 8.0. At pH 7.0 values of K for $GOase_{semi}$ are NCS^- (270 M^{-1}), N_3^- (4.9×10^3), and $CH_3CO_2^-$ (107).

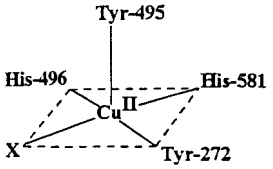
Galactose oxidase (GOase; EC 1.1.3.9) is a single Cu-containing two-equivalent oxidase (MW 68.5 kDa; 639 amino acids),[1,2] which in the active state uses Cu^{II} and a coordinated tyrosyl radical ($Tyr^•$) as redox-active components.[3−5] The two-equivalent oxidase activity with primary alcohols RCH_2OH can be expressed as in (1), and three GOase oxidation states defined

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

as in (2). X-ray crystal structures at resolutions down to 1.7 Å

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

have demonstrated that the Cu^{II} has a square-pyramidal geometry, with the radical forming Tyr-272, His-496, His-581, and the exogenous ligand X^- giving a square-planar geometry, and Tyr-495 as the axial ligand.[6−8] In the first structure acetate buffer



was incorporated at the X^- site at pH 4.5.[6] Crystals obtained from Pipes buffer at pH 7.0 have $X^- = H_2O$ or OH^-,[7] and the structure with $X^- = N_3^-$ has also been determined.[8] From EXAFS data it has been concluded that there is no significant change in the active site structure of $GOase_{ox}$ and $GOase_{semi}$.[9] In the catalytic cycle two-electron reduction of $GOase_{ox}$ with RCH_2OH is followed by the reaction with O_2, (3) and (4).

$$RCH_2OH + GOase_{ox} \xrightarrow{k_1} RCHO + GOase_{red}H_2 \qquad (3)$$

$$GOase_{red}H_2 + O_2 \xrightarrow{k_2} GOase_{ox} + H_2O_2 \qquad (4)$$

The reaction sequence is referred to as a "ping-pong" mechanism, where first one and then the other substrate binds to the Cu^{II}. The product written here as $GOase_{red}H_2$ denotes a doubly protonated species.[10,11] The Cu^{II} is ∼8 Å from the surface

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and is accessed via a channel. Reactions are stereoselective with respect to RCH_2OH, and whereas reaction with D-galactose is observed, there is no reaction with L-galactose.[12] From stopped-flow studies with five substrates, different k_1 values (1.1−2.9) $\times 10^4$ M^{-1} s^{-1} at pH 7.0, and a single k_2 value of 1.01×10^7 M^{-1} s^{-1}, independent of pH 5.5−9.0, have been determined.[10] Saturation kinetics for (3) have been studied with D-galactose and D-raffinose when Michaelis−Menten kinetics give K_M = 0.15 (and 0.07)/M, k_{cat} = 5500 (and 4200) s^{-1} at pH 7.0, with D-raffinose values in parentheses.[11] Values of $1/K_M$ give small substrate K_{bind} values (corresponding to K in the present work) of 6.7 (14.3) M^{-1}, respectively, consistent with GOase functioning as an extracellular enzyme. In vivo it has been suggested that GOase reacts with the primary alcohol group of carbohydrates and plays some part in lignin degradation.[12] The release of H_2O_2 is also believed to be relevant.[13]

In the present paper active site substitution is modeled using $X^- = NCS^-$, N_3^-, and $CH_3CO_2^-$ as nonredox active reagents, (5). Thermodynamic (K) and kinetic (k_f and k_b) parameters are

$$GOase_{ox} + X^- = GOase_{ox}, X^- \qquad (5)$$

reported. The pH dependencies help provide new insights and establish further the properties of the active site. Some comparisons are made with $GOase_{semi}$, although there is no evidence for this form being involved in the catalytic cycle (3) and (4). Unlike NCS^-, protonated forms of azide and acetate are known, with relevant acid dissociation pK_a values 4.64 for HN_3 at 25 °C, I = 0.10 M (KCl),[14] and 4.58 for CH_3CO_2H at 25 °C, I = 0.10 M (KNO_3).[15]

Methods and Materials

Isolation of Enzyme. GOase from the fungal source *Fusarium* (strain NRRL 2903)[16,17] was obtained using an *Aspergillus nidulans* (strain G191/pGOF101) expression system by a procedure already described.[18−20] The Tyr495Phe variant was obtained from a similar expression system.[21] Final purification was achieved using phosphocellulose column chromatography and confirmed by FPLC analysis.

Buffers. The following buffers were obtained from Sigma Chemicals or as stated: 2-(*N*-morpholino)ethanesulfonic acid (Mes, pK_a = 6.1, pH 5.5−6.7); sodium hydrogen phosphate (pK_a = 6.68 average for $H_2PO_4^-$,[22] pH 5.5−8.2); 2,6-dimethylpyridine, Fluka, (lutidine, pK_a 6.75, pH 5.7−8.0); 2-(*N*-cyclohexylamino)ethanesulfonic acid (Ches, pK_a 9.3, pH 8.6−10.0). All buffer solutions were prepared on the day of use or were stored ∼1 day at 4 °C to avoid microbial growth. The pH of solutions was checked using a Russell CWR-322 glass electrode and Radiometer PHM62 m.

None of the experiments with sodium acetate (Sigma Chemicals) were in the pH range where acetate functions as a buffer. Experiments with sodium hydrogen phosphates were included as a check on interactions with the GOase active site.[23−26] Experiments with NCS^-, N_3^-, and $CH_3CO_2^-$ in which 10 mM phosphate was used as buffer were repeated using Mes or lutidine at 10 mM. At this level the agreement of K values was satisfactory and within 5% of values obtained using phosphate buffer. It was also possible to exchange 50 mM phosphate by 50 mM lutidine buffer at pH 7.5 with good agreement (∼2%). However, effects of phosphate on rate constants for the autoreduction of $GOase_{ox}$ to $GOase_{semi}$ are observed as will be reported elsewhere.[27]

Preparation of Enzyme Solutions. Combined $GOase_{ox}$ and $GOase_{semi}$ concentrations were determined from the UV absorbance at 280 nm ($\epsilon = 1.05 \times 10^5$ M^{-1} cm^{-1}),[17] and the amount of catalytically competent Cu-containing protein was determined from the absorbance at 450 nm (ϵ = 8600 M^{-1} cm^{-1}) for $GOase_{ox}$ at pH 7.0. Newly prepared protein gave a Cu content of ∼90% as determined by the biquinoline colorimetric method.[28] Stored GOase samples were a mix of $GOase_{ox}$ and $GOase_{semi}$ (dominant form) due to autoredox processes. To convert the mix to $GOase_{ox}$ or $GOase_{semi}$, an excess of $K_3[Fe(CN)_6]$ (Sigma) or$K_4[Fe(CN)_6]\cdot 3H_2O$ (BDH Analar) respectively was added immediately prior to use. Excess of reagent and product were then removed in 1−2 min on a desalting P-6 DG column (Bio-Rad). Such rapid desalting techniques allow effects of WT $GOase_{ox}$ autoreduction to be minimized.[27] However, loss of Cu is observed on treating with $[Fe(CN)_6]^{3-/4-}$ (10−20% for each redox cycle depending on the procedure adopted and time taken), as has been observed previously for the Tyr495Phe variant.[29] Alternative redox reagents, e.g., $[Co(terpy)_2](ClO_4)_3$ as oxidant (terpy = 2,2′;6′,2″-terpyridine) or sodium ascorbate as reductant, can be used to avoid such effects.

To prepare reactant 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 with ionic strength adjusted to I = 0.100 M using NaCl. The dialysis solution was changed three times. Protein instability was observed at pH <5.5. All studies described were in air.

Titration Procedures for the Determination of *K*. Thiocyanate solutions (up to 0.100 M) were prepared from Na^+NCS^- (Fluka). Equilibrium constants K/M^{-1} for (6) were determined at 25.0 ± 0.1

$$GOase_{ox} + NCS^- \overset{K}{\rightleftharpoons} GOase_{ox}\ NCS^- \qquad (6)$$

°C by titration of NCS^- from a Gilson micropipet into $GOase_{ox}$. Buffer levels were 10 mM, and the ionic strength was retained at I = 0.100(1) M (NaCl), except as stated. UV−vis absorbance changes were instant, and absorbance (A) values at wavelengths 433 nm and/or 645 nm were monitored. At any one wavelength, (7) relates initial (A_0),

$$\frac{1}{A_{obs} - A_0} = \frac{1}{K(A_\infty - A_0)[NCS^-]} + \frac{1}{A_\infty - A_0} \qquad (7)$$

final (A_∞), and monitored (A_{obs}) absorbance values. Corrections of concentrations for dilution effects were made. Plots of $(A_{obs} - A_0)^{-1}$ against $[NCS^-]^{-1}$ were linear. From the ratio of intercept/slope values of K were evaluated. Good agreement in K values (variations <6%) at the two wavelengths was obtained, and average values are listed. Similar procedures were used for N_3^- and $CH_3CO_2^-$. Determination of K was

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restricted by the instability of the protein at pH <5.5. No effects on UV−vis spectra were observed on replacing 0.100 M levels of Cl^- with ClO_4^-.

Procedure for Kinetic Studies. The reactions of $GOase_{ox}$ with NCS^- and N_3^- were studied under the same conditions as for the determination of K. With $GOase_{semi}$ and $GOase_{ox}$ Tyr495Phe absorbance changes were too fast/too small for the reaction to be monitored. An Applied Photophysics SX-17MV stopped-flow reaction analyzer (dead time 1.2−1.5 ms) was used to monitor equilibration reactions with NCS^- (445 nm) and N_3^- (450 nm), present in >10-fold excess of GOase. Rate constants were obtained using SX-17MV software. Equilibration rate constants (k_{eq}) are an average of at least five traces for each run. pH-jump mixing was used at the lower pH's.

Electrospray Mass Spectrometry. The molecular weight (MW) of wild-type GOase used in these studies has not previously been checked. To do this, all traces of buffer were removed using a desalting column as above. Acetic acid (1%) was added immediately prior to introduction into a Micromass Autospec M spectrometer. The mobile phase was aqueous acetonitrile (60%), and cesium iodide was used as calibrant. A high nozzle skimmer cone voltage of 96.2 mV was required for detection. Mass spectra were obtained in the positive-ion mode, and analyzed using Micromass Opus software to give a mean MW of 68 653.6 Da, standard deviation 2.26. From X-ray crystallography the MW is reported as 68.5 kDa.[6,7]

Glassware. Standard cleaning procedures were employed using concentrated nitric acid followed by extensive rinsing with single-distilled water, further purified by deionizing on a Sanyo Fistreem column. Glassware cleaned by alternative procedures, including hexane followed by hot concentrated nitric acid,[23] gave identical results.

Treatment of Data. Computer fits, with parameters as indicated in the Results section, were used to draw the curved lines in Figures 5, 6, 7, 10, and 11.

Results

UV−Vis Determination of pK_a Values. Solutions of $GOase_{ox}$ (~15 μM) in 10 mM buffer were desalted, the unbuffered solution first adjusted to pH 4 by addition of 0.100 M HCl, and then titrated with 0.100 M NaOH (Gilson micropipet) to pH 9.1, I = 0.l00 M (NaCl). Absorbance changes were 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 $GOase_{ox}$ well-defined isosbestic points at 466 and 549 nm were observed for the pH range 4.0−6.5, and from the absorbance changes at a fixed wavelength pK_{1a} = 5.7(1), Figure 1A. Absorbance changes at higher pH's (6.5−9.1) gave no isosbestic points and pK_{2a} = 7.0(2), Figure 1B. Similar procedures were adopted for $GOase_{semi}$ and gave pK_{1a} = 5.6(2) and pK_{2a} = 8.0(2), Figure 2. No pK_{1a} was observed for $GOase_{ox}$ with NCS^- (0.4M) or N_3^- (5mM), when >98% anion coordination at the exogenous site is observed (see three sections below). For the variant $GOase_{ox}$ Tyr495Phe, pK_{1a} = 5.7(1), but no pK_{2a} was observed.

Reactions of $GOase_{ox}$ with NCS^-. UV−vis absorbance changes on titrating $GOase_{ox}$ with NCS^- are illustrated in Figure 3. At pH 7.5 isosbestic points were observed at 390, 506, and 549 nm. From linear plots of $(A_{obs} - A_0)^{-1}$ vs $[NCS^-]^{-1}$, eq 6, the ratio of intercept/slope gives equilibrium constants K at different pH's (Table S1 of the Supporting Information). At pH 7.0, $K = 0.49(2) \times 10^3$ M^{-1}, with standard deviations (~4%) typical of individual determinations.

Stopped-flow studies with NCS^- in >10-fold excess gave equilibration rate constants, k_{eq}. Forward and back rate constants are as defined in (8). Linear plots of k_{eq} vs $[NCS^-]$ are consistent

$$GOase_{ox} + NCS^- \underset{k_b}{\overset{k_f}{\rightleftharpoons}} GOase_{ox}\,NCS^- \qquad (8)$$

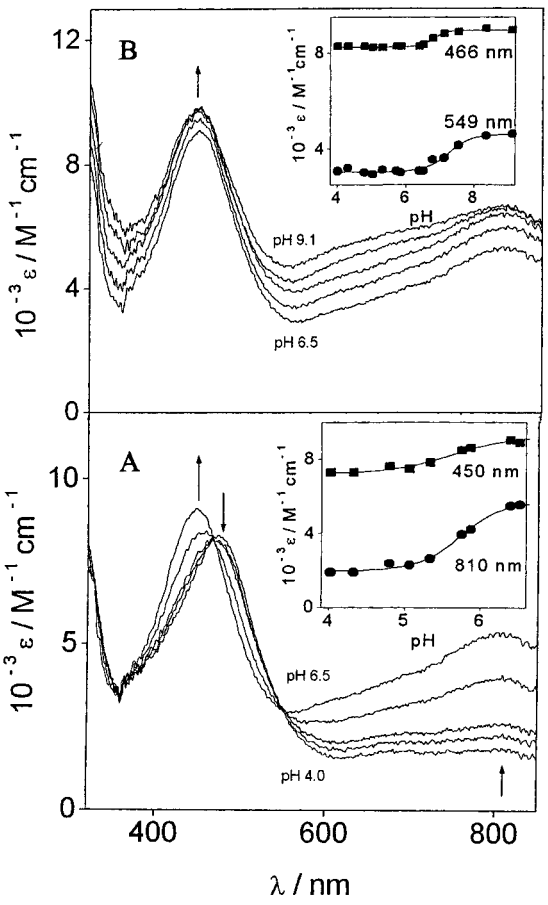


Figure 1. Effect of increasing pH from 4.0 to 9.1 on UV−vis spectra (25 °C) for WT $GOase_{ox}$ (~10 μM) with no buffer present. Two separate pH ranges A and B are indicated defining pK_{1a} and pK_{2a} respectively (see inset), I = 0.100 M (NaCl).

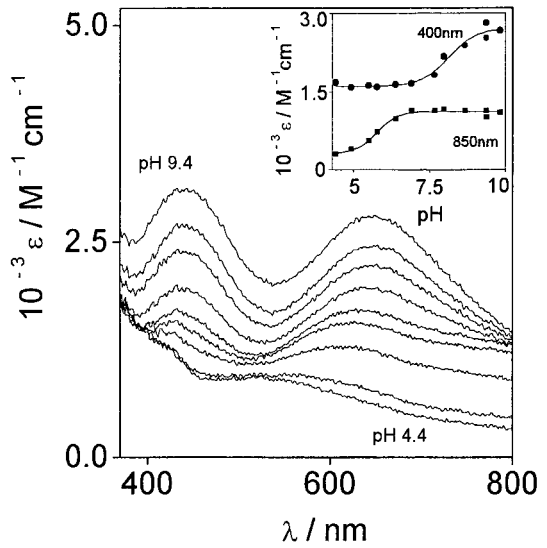


Figure 2. Effect of increasing pH from 3.9 to 9.4 on the UV−vis absorbance spectrum of WT $GOase_{semi}$ (~30 μM) with no buffer present at 25 °C, I = 0.100 M (NaCl). Two pH ranges are apparent defining pK_{1a} and pK_{2a}.

with (9), Figure 4, and give k_f (slope) and k_b (intercept) values.

$$k_{eq} = k_f[NCS^-] + k_b \qquad (9)$$

Variations in k_f and k_b (inset) with pH are illustrated in Figure 5, and values are listed in Table S2 of the Supporting

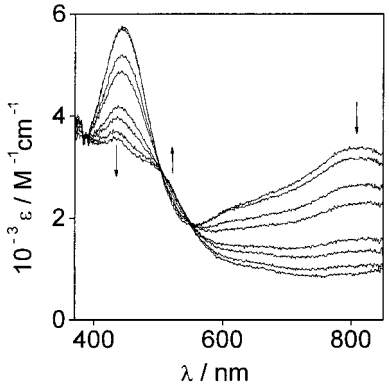


Figure 3. UV−vis spectra (25 °C) for the titration of NCS^- (microsyringe) with WT $GOase_{ox}$ at pH 7.5, $I = 0.100$ M (NaCl). Equilibration is rapid on addition of each aliquot of NCS^-.

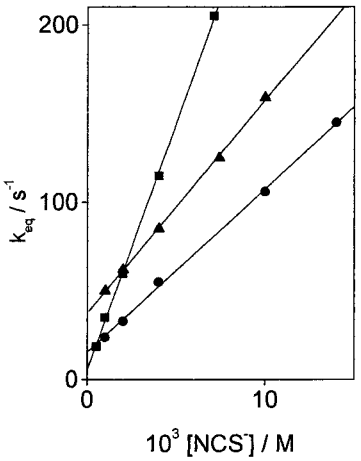


Figure 4. Dependence of equilibration rate constants k_{eq}(25 °C) on [NCS^-] for the stopped-flow reaction of NCS^- with WT $GOase_{ox}$ at pH 5.5 (■); 6.5 (●); 7.9 (▲); $I = 0.100$ M (NaCl).

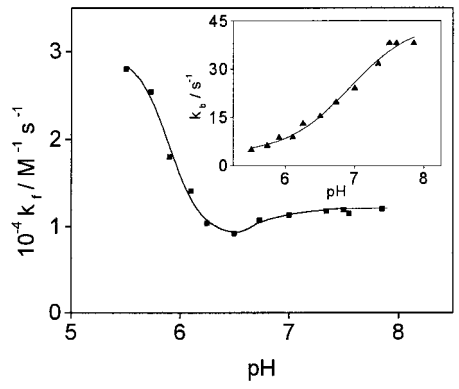


Figure 5. Variation of formation and back rate constants, k_f and k_b (25 °C), respectively, with pH for the reaction of NCS^- with $GOase_{ox}$, $I = 0.100$ M (NaCl).

Information. In the case of k_f both pK_{1a} and pK_{2a} are seen to be influential. The variation of k_b with pH is attributed to the process pK_{2a} (there is no coordinated H_2O, and therefore no pK_{1a}). Values of K ($=k_f/k_b$) alongside those from UV−vis titrations are also shown in Table S1, and the pH dependence of all K values is illustrated in Figures 6 and 7. Scheme 1 summarizes these different reactions. At pH 7.0, K for kinetic studies is $0.47(5) \times 10^3$ M^{-1}, in excellent agreement with the value (0.49×10^3 M^{-1}) from titration procedures. The higher standard deviations for K values from kinetic studies (in this study ~10%) are due to the small intercepts k_b in (9). From

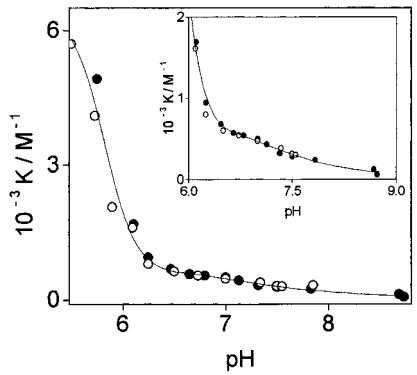


Figure 6. Variation of equilibration constants K(25 °C) with pH, $I = 0.100$ M (NaCl), for the reaction of NCS^- with WT $GOase_{ox}$ from (a) UV−vis titrations as in Figure 3 (●) and (b) kinetic studies (○). Both pK_{1a} and pK_{2a} are influential. The inset shows an enlarged version of the region which defines pK_{2a}.

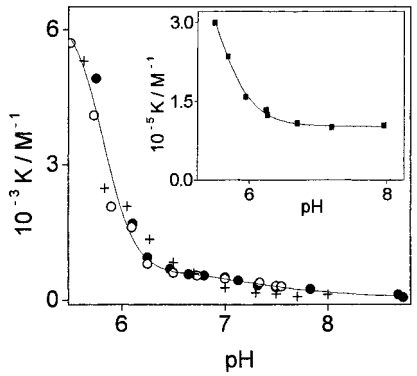
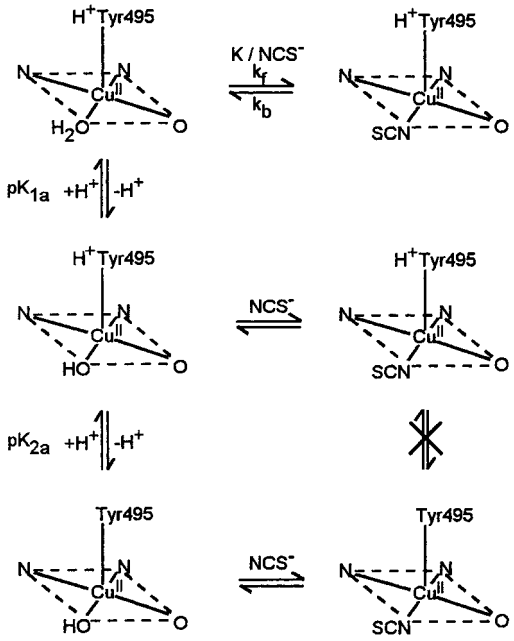


Figure 7. Comparison of equilibrium constants K(25 °C) for the reaction of WT $GOase_{ox}$ (●, ○) as in Figure 6, and WT $GOase_{semi}$ (+) with NCS^-, $I = 0.100$ M (NaCl). The inset shows the variation of equilibrium constants K for the corresponding reaction of $GOase_{ox}$ Tyr495Phe (■) with NCS^-. Only pK_{1a} is observed in the latter case.

Scheme 1



these variations pK_{1a} is 5.8(3) and pK_{2a} is 7.0(2) in agreement with the spectrophotometric values of 5.7 and 7.0.

UV−vis absorbance changes for titration of the $GOase_{ox}$ Tyr495Phe variant with NCS^- were carried out, and K values

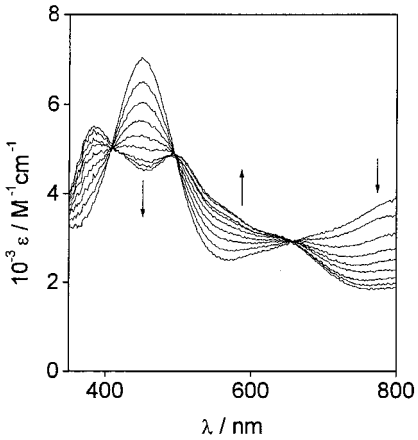


Figure 8. UV−vis spectra (25 °C) for the titration of N_3^- (microsyringe) to a solution of WT $GOase_{ox}$ (~18 μM) at pH 7.5, $I = 0.100$ M (NaCl). Equilibration is rapid on addition of each aliquot of N_3^-.

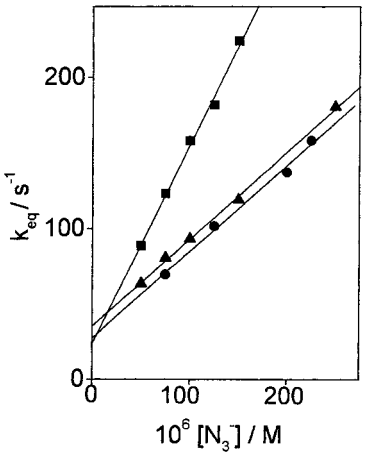


Figure 9. Dependence of equilibration rate constants k_{eq}(25 °C) on [N_3^-] for the stopped-flow reaction of N_3^- with WT $GOase_{ox}$ at pH 6.0 (■), 7.0 (●), 7.9 (▲), $I = 0.100$ M (NaCl).

obtained, Table S1. The variation of K with pH gives $pK_{1a} < 6.0$ in keeping with the spectrophotometric value of 5.7, assigned as acid dissociation of coordinated H_2O; see the inset to Figure 7. No variations corresponding to pK_{2a} were observed. On this and other evidence,[19] pK_{2a} can be assigned as acid dissociation of $Tyr495H^+$. The axial Cu^{II}−O (Tyr-495) bond of wild-type $GOase_{ox}$ is long due to a Jahn−Teller distortion.[6−8] Substantial catalytic activity of $GOase_{ox}$ is retained at pH 5.5 when protonation of Tyr-495 is approaching completion.[10,11] Since $GOase_{ox}$ Tyr495Phe does not exhibit catalytic activity, this suggests that protonation of Tyr-495 does not result in its dissociation from the Cu^{II}.

Reaction of $GOase_{ox}$ with N_3^-. From X-ray crystallography N_3^- is known to coordinate as X^- at the substrate binding site.[8] It has been demonstrated that N_3^- and the dihydroxyacetone substrate bind at the same site.[9] UV−vis absorbance changes are illustrated in Figure 8 for the titration of $GOase_{ox}$ with N_3^-. At pH 7.0 isosbestic points are observed at 411, 483, and 650 nm, and using the same procedure as for NCS^-, $K = 1.92(4) \times 10^4$ M^{-1}, where the standard deviation is ±2%. Stopped-flow studies with N_3^- in >10-fold excess gave equilibrium rate constants k_{eq}. From the graphical treatment, Figure 9, k_f (slope) and k_b (intercept) values were obtained at pH 6.00, 7.00, and 7.92, and are listed in Table S3 of the Supporting Information. At pH 7.0, the kinetic K ($=k_f/k_b$) is $2.04(3) \times 10^4$ M^{-1}, in good agreement with the titration value. While k_f is 45 times bigger than that for the NCS^- reaction, k_b is very similar (6% bigger)

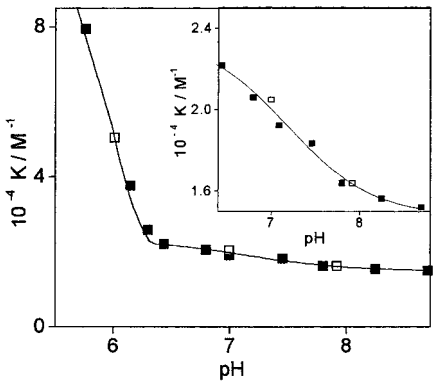


Figure 10. Variation of equilibration constants K(25 °C) with pH, $I = 0.100$ M (NaCl), for the reaction of N_3^- with WT $GOase_{ox}$ from (a) UV−vis titrations (■) and (b) kinetic studies (□). Two pK_a's are indicated. The region which defines pK_{2a} is shown enlarged in the inset.

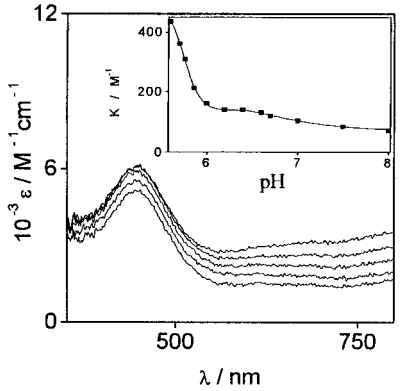


Figure 11. Determination of K(25 °C) from UV−vis spectrophotometric changes on titration of $GOase_{ox}$ (~10 μM) with acetate (microsyringe) at pH = 7.5, $I = 0.100$ M (NaCl). Inset shows the variation of K with pH.

at pH 7.0. The pH variations of K from both UV−vis and kinetic measurements are illustrated in Figure 10. Individual K values are ~40 times greater for N_3^- than NCS^-, Table S4 of the Supporting Information. From the variations of K with pH, $pK_{1a} < 6$ and $pK_{2a} = 7.2(1)$.

Effect of Coordinated NCS^- and N_3^- on pK_{2a} for $GOase_{ox}$. From K(25 °C) values for the coordination of NCS^- and N_3^- to $GOase_{ox}$, Figures 6 and 10, it was calculated that 0.4 M NCS^- or 5 mM N_3^- gives >98% anion binding at the H_2O site on Cu^{II} at all pH's relevant to this section. $GOase_{ox}$ solutions at pH 5.76 and 8.05 were prepared, and the anion concentration was adjusted to the required levels. On changing the pH from 5.76 to 8.05 or vice versa, no change in UV−vis absorption corresponding to reequilibration was observed. Therefore H^+ is not able to leave or access the Tyr-495 site when one or the other of the anions is in place.

Reaction of $GOase_{ox}$ with $CH_3CO_2^-$. From X-ray crystallography acetate is also known to coordinate at the substrate binding site X.[6] The pH range 5.6−8.0 studied was outside the buffering range for acetate. In this range the acetate anion is present. From UV−vis scan spectra with increasing acetate there are no isosbestic points, Figure 11. Values of K were obtained from absorbance changes at a fixed wavelength, and from the variations with pH, Table S4 of the Supporting Information, $pK_{1a} = 5.8(1)$ and $pK_{2a} = 6.9(2)$. At pH 7.0 a value of K(25 °C) = 104(4) M^{-1} was obtained, indicating standard deviations of ~4%.

Reaction of $GOase_{semi}$ with NCS^-. The same UV−vis spectrophotometric procedures were used to determine K for

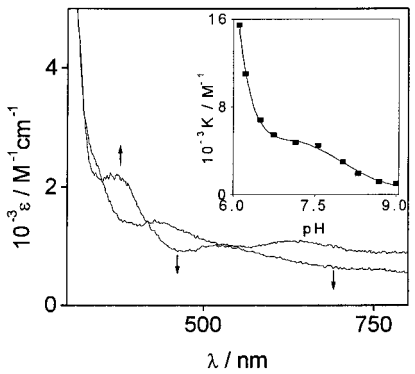


Figure 12. UV−vis spectrophotometric changes for the titration of N_3^- with $GOase_{semi}$ (82 μM) to determine the equilibrium constant K(25 °C) at pH 7.56. Initial and final spectra only are shown for clarity. The variation of K with pH is also shown (inset).

$GOase_{semi}$ at different pH's. At pH 7.0 an isosbestic point at 395 nm was observed, and at pH 7.0, $K = 2.7(1) \times 10^2$ M^{-1}. Values of K are included in Table S1, and in Figure 7 alongside those for $GOase_{ox}$. Differences in K for $GOase_{ox}$ and $GOase_{semi}$ are small, indicating a controlling influence of the Cu^{II} in each case. On pH titration of $GOase_{semi}$ (no buffer present), acid dissociation constants are $pK_{1a} = 5.6(2)$, and $pK_{2a} = 8.0(2)$, Figure 2. The response of K to pK_{1a} is clearly illustrated in Figure 7. The response to pK_{2a} is less well defined, possibly because absorbance changes are small in the pH range 6.8−8.0. Stopped-flow kinetic studies were attempted, but proved too fast for the small absorbance changes.

Reaction of $GOase_{semi}$ with N_3^-. The same procedure as above was used to determine K at different pH's. At pH 7.56 two isosbestic points are observed, Figure 12. Values of K are included in Table S5 of the Supporting Information. At pH 7.0, $K = 0.50(2) \times 10^4$ M^{-1}. The variation of K with pH is shown in the inset to Figure 12, which defines $pK_{1a} < 6$ and $pK_{2a} = 8.1(1)$. Both pK_a's are in accordance with values determined by UV−vis spectrophotometry, Figure 2. The value of pK_{2a} is ascribed to protonation of the axial Tyr-495 ligand. Stopped-flow rate constants could not be determined.

Discussion

In this work it has been demonstrated from UV−vis spectrophotometry that the $GOase_{ox}$ and $GOase_{semi}$ active sites have two acid dissociation constants K_{1a} and K_{2a}. Distinguishing features in the case of $GOase_{ox}$ are the retention of two isosbestic points in UV−vis spectra at pH 4.1−6.5, which help define the acid dissociation constant K_{1a} (hence pK_{1a}). Over the second part of the pH range 6.5−9.1 no isosbestic points are observed, and the pH changes define pK_{2a}. The pK_{1a} process is not observed when X^- is present, and is assigned as acid dissociation of the coordinated H_2O, (10). No pK_{2a} value is evident for the

$$Cu^{II}-OH_2 \overset{K_{1a}}{\rightleftharpoons} Cu^{II}-OH^- + H^+ \qquad (10)$$

Tyr494Phe variant, and pK_{2a} is assigned to H^+Tyr-495, (11).

$$Cu^{II}-H^+Tyr495 \overset{K_{2a}}{\rightleftharpoons} Cu^{II}-Tyr495 + H^+ \qquad (11)$$

Noninteracting buffers, i.e., Mes, lutidine, and Ches,[20] were used, and with phosphate amounts were kept at low levels. Previously the reported uptake of 1 equiv of H^+ on binding NCS^- and N_3^- to $GOase_{ox}$, and N_3^- to $GOase_{semi}$, was explained by an accompanying protonation of Tyr-495.[21] However, at pH >6.0 (a pH of 8.0 was used in ref 24) an alternative explanation is that pK_{1a} occurs, (10), and is followed by anion displacement of OH^- with concomitant H^+ uptake.

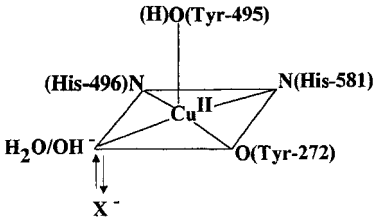


Figure 13. Summary of different reactions occurring at the $GOase_{ox}$ and $GOase_{semi}$ active sites.

Table 1. Acid Dissociation Constants (25 °C) for $GOase_{ox}$ and $GOase_{semi}$, Where pK_{1a} Is Assigned to the Coordinated H_2O and pK_{2a} to H^+Tyr-495[a]

protein	procedure	pK_{1a}	pK_{2a}
WT $GOase_{ox}$	UV−vis[b]	5.7(1)	7.0(2)
	UV−vis[c]		6.7(2)[d]
	K(NCS^-)	5.8(1)	7.0(2)
	K(N_3^-)	<6.0	7.0(1)
	K($CH_3CO_2^-$)	5.8(1)	6.9(2)
Tyr495Phe $GOase_{ox}$	UV−vis[b]	<6.0	
	K(NCS^-)	<6.0	
WT $GOase_{semi}$	UV−vis[b]	5.8(1)	8.1(2)
	UV−vis[c]		7.9(2)[d]
	K(NCS^-)	5.8(1)	
	K(N_3^-)	<6.0	8.1(1)
	K($CH_3CO_2^-$)	5.7(1)	7.9(1)
	redox kinetics[e]		8.0(2)[d]
Trp290His $GOase_{semi}$	UV−vis[c]		6.9(3)[f]

[a] The procedures referred to are UV−vis buffer-free pH titrations, conventional pH titrations with buffers present, the variations of formation constants K with pH for NCS^-, N_3^-, $CH_3CO_2^-$, and rate constant redox studies with pH, $I = 0.100$ M (NaCl). Standard deviations are indicated in parentheses. [b] Buffer-free titrations. [c] pH variations in the presence of buffers, studies carried out prior to pK_{1a} assignment. [d] Reference 19. [e] Average of values in range 7.9−8.1. [f] Reference 20.

Table 2. Summary of Equilibrium Constants K and Rate Constant k_f and k_b Data (25 °C) for the Reactions of NCS−, N_3−, and CH_3CO_2− with Different GOase Forms at pH 7.0, $I = 0.100$ M (NaCl)[a]

reaction	K/M^{-1}	k_f/M^{-1} s^{-1}	k_b/s^{-1}
WT $GOase_{ox} + NCS^-$	$0.47(5) \times 10^3$	$1.13(4) \times 10^4$	24(2)
WT $GOase_{ox} + N_3^-$	$2.04(3) \times 10^4$	$5.20(2) \times 10^5$	25.5(3)
WT $GOase_{ox} + CH_3CO_2^-$	104(4)		
Tyr495Phe $GOase_{ox} + NCS^-$	1.0×10^5	*b*	*b*
WT $GOase_{semi} + NCS^-$	$0.27(1) \times 10^3$	*b*	*b*
WT $GOase_{semi} + N_3^-$	$5.0(2) \times 10^3$	*b*	*b*
WT $GOase_{semi} + CH_3CO_2^-$	107(5)		

[a] Standard deviations are indicated in parentheses. [b] Fast process with small absorbance changes.

UV−vis spectrophotometric changes are observed for the complexing of $X^- = NCS^-$, N_3^-, and $CH_3CO_2^-$ to $GOase_{ox}$, and formation constants K have been determined. From X-ray crystal structures N_3^- and $CH_3CO_2^-$ are known to complex at the Cu^{II} substrate binding site,[6−8] and with NCS^- the corresponding reaction is assumed to occur. The various reactions are summarized in Figure 13. As the pH is varied, both pK_{1a} and pK_{2a} are seen to influence K and provide a further means of determining the pK_a's. Table 1 provides a summary of pK_{1a} and pK_{2a} for $GOase_{ox}$ and $GOase_{semi}$ from different procedures, and Table 2 provides a summary of K values for different X^- ligands. The scheme for $GOase_{ox} + NCS^-$ applies also to $GOase_{semi}$ and for all three anions. Whereas pK_{1a} can be regarded as identical for $GOase_{ox}$ (5.7) and $GOase_{semi}$ (5.6), which is not

unreasonable since Cu^{II} is present in both cases, pK_{2a} shows a significant shift from 7.0 to 8.0. This indicates an influence of Tyr-272 (and whether it is present as a radical) on the extent of protonation of Tyr-495. Such an observation is in keeping with the ~800 nm absorbance of $GOase_{ox}$, which has been assigned as an interligand Tyr-495 → Tyr-272 (radical) charge-transfer band.[2]

Values of K/M^{-1} at pH 7.0 for NCS^- (480), N_3^- (1.98×10^4), and $CH_3CO_2^-$ (104) indicate a 40-fold more effective reaction of N_3^- as compared to NCS^-. From literature stability constant data for hexaaqua Cu^{II}, there is no clear indication that formation constants for reactions with N_3^- are more favorable than those for NCS^-.[30] The decreased affinity of NCS^- for the Cu^{II} of GOase is possibly due to the greater difficulty NCS^- (with its bigger S-atom) has in accessing the active site via the ~8 Å channel. The carboxylate of $CH_3CO_2^-$ is also bulkier and has less affinity for Cu^{II} at the active site. The 200-fold bigger K for the $GOase_{ox}$ Tyr495Phe reaction with NCS^- at pH 7.0 is attributed to the smaller coordination number of 4 instead of 5 when phenylalanine is present. Crystals of the Tyr495Phe variant have been produced which diffract to 2.8 Å.[21] The difference map as compared to the wild-type structure[6,7] shows a slight shift in the Tyr-495 ring position, a compensating movement in the ring of His-495, and small shift of Tyr-272. Values of K/M^{-1} for D-galactose (6.7) and D-raffinose (14.3) at pH 7.0 are small,[11] which can also be attributed to size and (depending on the pK_a of the alcohol RCH_2OH group) the absence of a favorable negative charge. In work of Kimura and colleagues pK_a values of ~7.4 have been reported for ligands with a pendant $-CH_2OH$ group,[31,32] and are a guide as to values expected for the RCH_2OH substrates. Not surprisingly, K and k_f for anionic species such as NCS^- or N_3^- are both smaller on replacing the Cu^{II}-coordinated H_2O by OH^-, due to the less favorable electrostatic interactions. Whereas K decreases further on deprotonation of Tyr-495, in keeping with the decrease in net charge of the active site, k_b increases possibly due to the decreased electrostatic attraction for the coordinated anion. Rate constants $k_f/M^{-1}\ s^{-1}$ at pH 7.0 for NCS^- (1.13×10^4) and N_3^- (5.2×10^5) are similar to values obtained for five primary alcohol substrates, which are in the range $(1.1-2.9) \times 10^4\ M^{-1}\ s^{-1}$ at pH 7.5 and above.[10] Values of k_b for NCS^- and N_3^- are essentially the same, which in terms of a dissociative process suggests similar $Cu^{II}-X$ bond strengths. Although $GOase_{semi}$ has been less extensively studied, there are strong similarities in K values to those for $GOase_{ox}$ (Table 2), largely determined by the presence of Cu^{II} in both cases.

From knowledge of K values it is possible to calculate concentrations of NCS^- (0.4 M) or N_3^- (5.0 mM) required to give >98% complexing at the Cu^{II} active site over selected pH ranges. On changing the pH of such solutions from 5.76 to 8.05 (and vice versa), negligible change in the UV−vis spectrum was observed, indicating that X^- blocks protons leaving or accessing the Tyr-495 site.

The enzyme mechanism requires updating in the light of these new findings, in particular the unusually small pK_a observed for the acid dissociation of the exogenous H_2O. A key part of the mechanism of the 2-equiv reduction of primary alcohols RCH_2OH,[10,33,34] is the initial interaction of $GOase_{ox}$ with substrate, when both pK_{1a} and pK_{2a} are able to exert an influence. Stage 1 involves the coordination of RCH_2OH, possibly as RCH_2O^- (depending on the pK_a)[31,32] to the Cu^{II}. If Tyr-495 is not protonated, some proton transfer from RCH_2OH may occur. In other words a total of zero, one, or two protons can be attached at the Tyr-495 and RCH_2O^- sites depending on the pH at the outset. Stage 2 involves H-atom transfer from the substrate CH_2 to the coordinated (unprotonated) Tyr-272 radical and further transfer of an electron to the Cu^{II}.[16] Isotopic labeling with α-galactopyranoside as substrate indicates a large isotope effect ($k_H/k_D \sim 20$), consistent with homolytic cleavage of the methylene C−H bond and H-atom transfer to the radical at Tyr-272.[25] Proton uptake by the active site prepares the way for a change in Cu^I coordination number to 2, 3, or 4 (all of which are more acceptable than 5 for a d^{10} ion).

To summarize, aqueous solution properties of $GOase_{ox}$ and $GOase_{semi}$ have been studied. A number of different GOase reactions and side reactions have been quantified. Processes identified include the active site acid dissociation pK_{1a} (of coordinated H_2O) and pK_{2a} (of H^+Tyr-495), both effective in the pH range 5.5−9.0. Side reactions include autoredox interconversion of $GOase_{ox}$ and $GOase_{semi}$, to be addressed elsewhere,[29] which is effective over ~4 h periods. In addition, the use of $[Fe(CN)_6]^{3-}$ to generate $GOase_{ox}$, and $[Fe(CN)_6]^{4-}$ for $GOase_{semi}$, results in the removal of Cu (~10% for each cycle) with formation of an apo-GOase component. The main reactions studied have been the determination of 1:1 formation and rate constants for the reactions of NCS^-, N_3^-, and $CH_3CO_2^-$ with $GOase_{ox}$ and $GOase_{semi}$. The studies provide information relevant to substrate binding and processes occurring in the GOase catalytic cycle. Coordinated anions are found to block protonation and deprotonation of Tyr-495.

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Supporting Information Available: Listing of thermodynamic and kinetic data (6 pages). This material is available free of charge via the Internet at http://pubs.acs.org.

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