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## Articles

# Redox Activation of Galactose Oxidase: Thin-Layer Electrochemical Study

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ABSTRACT: The redox activation of galactose oxidase by various oxidants is characterized by using a unique thin-layer electrochemical cell. Activation is shown to be strictly a redox process and can be rapidly accomplished by using ferricyanide, cobalt terpyridine or tetracyanomonophenanthroline ferrate, and a control electrode to control solution potential. This oxidation is a one-electron process and does not result in modified galactose oxidase which exhibits enhanced activity. On the contrary, this oxidation is required for activity. The solution potential dependence of activity is independent of which of these mediator–titrants is used, the concentration used, and which of various substrates is used in the determination. The substrates used were acetol, dihydroxyacetone, glycerin, 2-propyn-1-ol, allyl alcohol, 2-butyne-1,4-diol, furfuryl alcohol, benzyl alcohol, 4-pyridylcarbinol, galactose, and stachyose. The $E_{1/2}$ and $n$ values obtained are 0.40 ± 0.005 V vs. SHE and 0.9 ± 0.1 electron at pH 7.3. $E_{1/2}$ is defined as the potential at which half the maximal enzymatic activity is observed and probably reflects the $E^{0\prime}$ of the enzymic group involved in activation. A model is proposed in which activation occurs during turnover due to the redox buffering (by oxidants) of an enzymic Cu(II)/Cu(I) state which has a higher $E^{0\prime}$ than in resting galactose oxidase. The pH dependence of $E_{1/2}$ is 60 mV/pH unit in the pH range 6.0–8.0. The data suggest that the deprotonation of an amino acid residue facilitates the one-electron oxidation (activation) of galactose oxidase.

Galactose oxidase (EC 1.3.3.9) is the only known enzyme that contains one type 2 Cu(II) and no other prosthetic group. By use of this single Cu site, it catalyzes the two-electron oxidation of a variety of primary alcohols (Ettinger & Kosman, 1981).

$$RCH_2OH + O_2 \rightarrow RCHO + H_2O_2$$

Under the appropriate conditions it can utilize a number of oxidants including ferricyanide, porphyrexide, and iridium chloride in place of oxygen (Ettinger & Kosman, 1981). Under aerobic conditions, these and other oxidants activate galactose oxidase by as much as 30 times (Hamilton et al., 1978). The mechanism of this activation is unknown and is an important question with implications concerning the catalytic mechanism.

The major drawback associated with previous studies concerning activation is that in all cases there was no direct means by which to control solution potential in order that the effects of specific oxidants could be separated from those of changing the solution potential. This is a problem, since the solution potential resulting from the introduction of a given oxidant is dependent upon the concentration of the oxidant and its formal potential as well as those of other redox active species present. This problem is also particularly troublesome when enzyme assays or kinetic experiments are attempted and the substrates and/or products are redox active.

The purpose of this study was to investigate more completely the redox properties of galactose oxidase and, in particular, the mechanism of activation by various oxidants. A unique thin-layer cell containing immobilized galactose oxidase and a gold electrode to directly control solution potential was used (Johnson et al., 1982a). Relative enzyme activity as determined by $H_2O_2$ production was measured at a separate electrode. Relative activity is defined as the steady-state response of the $H_2O_2$ electrode and is proportional to absolute activity (Mell & Maloy, 1975). This cell allowed the determination of relative activity as a function of solution potential by using various mediators (Johnson et al., 1983) to mediate potential

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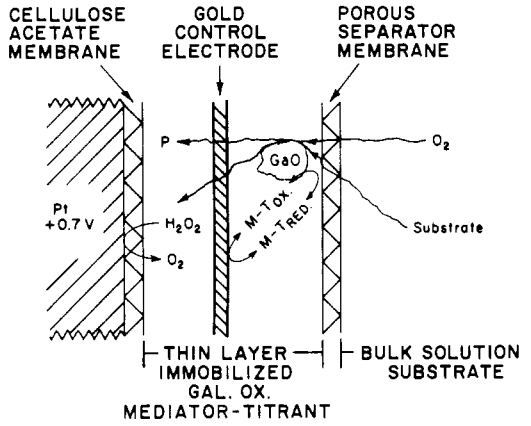



FIGURE 1: Schematic representation of the cell showing the Pt-working electrode used for peroxide measurement and the gold-working electrode used for control of the redox state of the enzyme. Galactose oxidase is immobilized on the Au-working electrode and entrapped between the two permeable membranes. The $O_2$ concentration is held relatively constant during turnover at 1.2 mM (atmospheric) which is about 30% saturated.

control to the enzyme during turnover.

In a previous report we described this cell and presented data that supported the conclusion that it could indeed be used to study the redox properties of galactose oxidase as long as the enzyme activity in the membrane (see Figure 1) was no more than about 0.6 international unit (IU)/$\mu$L. Under these conditions the response of the $H_2O_2$ electrodes is limited by the rate of the enzyme reaction and therefore reflects relative enzyme activity undistorted by the effects of diffusion (Johnson et al., 1982a). Immobilized galactose oxidase is necessary in order to obtain the enzyme-limited condition and yet retain the enzyme within the thin-layer cell. It was concluded that the dependence of galactose oxidase activity upon solution potential is relatively unaffected by immobilization with glutaraldehyde since (1) the midpoint potential ($E_{1/2}$) obtained was independent of the molecular ratio of glutaraldehyde to enzyme over a range of 100:1 to 6:1, (2) the same $E_{1/2}$ was obtained when dimethyl suberimidate was used to immobilize galactose oxidase, and finally, (3) the $E_{1/2}$ obtained by using immobilized galactose oxidase agreed quite closely with that obtained by using enzyme free in solution and simply adjusting solution potential by changing the ratio of ferricyanide to ferrocyanide (Johnson et al., 1982a; Dyrkacz et al., 1976). We also reported that the $E_{1/2}$ and $n$ values obtained for galactose oxidase were independent of the substrate concentration used in the determination (Johnson et al., 1982a). We report here the dependence of activation upon pH, mediator, mediator concentration, and substrate and discuss the importance of these results with regard to the catalytic mechanism of galactose oxidase.

## Experimental Procedures

*Cell Description.* Figure 1 is a schematic representation of the cell. The two working electrodes used in the cell are shown; the platinum-working electrode (Pt) is used for the amperometric determination of $H_2O_2$ and the gold control electrode for the control of solution potential within the enzyme layer. Control of the enzyme oxidation state is therefore mediated by the mediator–titrant redox couple. When the steady-state hydrogen peroxide production is measured with the platinum electrode behind this membrane assembly, the relative steady-state activity of galactose oxidase in the thin layer can be determined in the presence of a substrate that is introduced into the bulk solution and allowed to equilibrate across the outer separator membrane. This technique allows the simultaneous control of solution potential within the thin layer and measurement of relative enzymatic activity of the trapped enzyme. A more complete description of the cell (configuration II) and its construction can be found in Johnson et al. (1982a).

*Measurement of $E_{1/2}$.* For any given substrate the $E_{1/2}$ was measured by (A) setting a potential of $\sim$ +300 mV vs. Ag/AgCl on the gold control electrode, (B) injecting 25 $\mu$L of the substrate solution into the cell, (C) allowing the $H_2O_2$ current to reach a steady state, and then (D) stepping the potential of the gold control electrode negatively by 20-mV increments through $E_{1/2}$ while recording the steady-state $H_2O_2$ current at each new applied potential. This procedure was then reversed and the average taken of the $E_{1/2}$ for each direction. At each new potential a wait of 1–3 min was required before the $H_2O_2$ current reached a new steady state. The potential of the Ag/AgCl reference for the gold control electrode was monitored by using an SCE electrode in the supply bottle. By use of the $H_2O_2$ current data, the ratio of oxidized to reduced enzyme could be calculated at each applied potential since the reduced form is inactive. Least-squares regression analysis was used to fit these data to the Nernst equation, yielding $E_{1/2}$. All measurements were done at 25 °C.

*Apparatus.* The apparatus used in this study was the same as that described previously (Johnson et al., 1982a).

*Reagents.* All reagents were analytical grade unless specified otherwise. Acetol, benzyl alcohol, furfuryl alcohol, 4-pyridylcarbinol, allyl alcohol, and 2-butyne-1,4-diol were purchased from Aldrich Chemical Co. Eastman Chemical Co. was the source of 2-propyn-1-ol. Sodium citrate, sodium nitrate, potassium ferricyanide, and potassium ferrocyanide were obtained from Fisher Scientific Co. Sodium barbital was obtained from Mallinckrodt Chemical Co. Tetracyanomonophenanthroline ferrate and cobalt terpyridine were prepared according to published procedures (Schilt, 1960; Baker et al., 1959). Galactose oxidase was isolated and purified from cultures of *Dactilium dendroides* by the procedure of Kosman et al. (1974). The specific activity of the purified enzyme was approximately 2000 IU/mg (coupled assay), and pulsed power electrophoresis was performed as described earlier with essentially the same results, which indicated that the enzyme preparation used contained approximately five isoenzymes and no other protein (Kosman et al., 1974).

*Bulk Solution.* Except for the pH studies, the bulk solution in which membranes were stored and measurements taken was a pH 7.3, 0.07 M phosphate buffer which was 1 mM in mediator–titrant (unless otherwise noted) and 0.05 M in sodium chloride. Citrate–phosphate buffers were used in all pH studies up to and including pH 7.5, and sodium barbital buffers were used above pH 7.5. These buffers also contained 0.05 M NaCl, and the ionic strength was maintained constant ($\mu$ = 0.2) with sodium nitrate.

*Studies on Free Enzyme.* Free galactose oxidase assays used a Yellow Springs Instrument Co. Model 27 industrial analyzer to measure hydrogen peroxide. Enzyme solutions were injected into the thermostated (37 °C) sample chamber which contained a 0.07 M, pH 7.3, phosphate buffer, 0.05 M in NaCl and 0.7 M in galactose (Johnson et al., 1981). The final weight of enzyme present was $10^{-4}$–$10^{-5}$ mg. All dilutions of galactose oxidase were made into a buffered 2 mg/mL bovine serum albumin solution.

## Results

Figure 2 is an "activity voltammogram" for galactose and dihydroxyacetone taken by using a membrane containing about

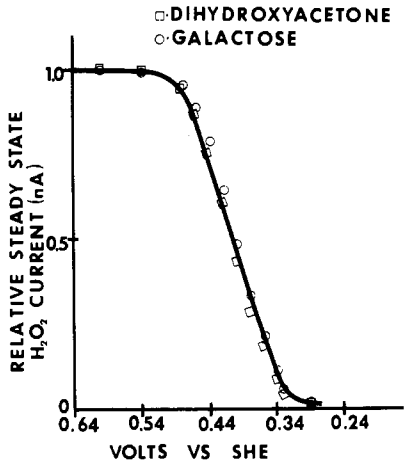



FIGURE 2: Potentiostatic activity voltammograms for galactose (○) and dihydroxyacetone (□) determined on a typical 0.6 IU/μL galactose oxidase membrane. The mediator–titrant was 1 mM $K_2Fe(phen)(CN)_4$.

0.6 IU/μL galactose oxidase and illustrates that under these conditions the dependence of enzyme activity upon solution potential is essentially the same whether galactose or dihydroxyacetone is used in the determination (Johnson et al., 1982a,b). Least-squares fitting of the data to the Nernst equation gave $E_{app} = 0.408 + 0.02794 \ln [(O)/(R)]$ for dihydroxyacetone and $E_{app}$ v $0.400 + 0.02803 \ln [(O)/(R)]$ for galactose. The correlation coefficients were 0.997 and 0.998, respectively.

As previously reported, essentially the same activity dependence upon solution potential is observed by using enzyme free in solution and varying ratios of ferri- to ferrocyanide to control potential (Johnson et al., 1982a). However, to further verify that the free enzyme could indeed be essentially entirely turned off at potentials around +0.24 V vs. SHE, some assays were performed with $Co(terpy)_2Cl_2$ to chemically control solution potential in this range. In the absence of any solution potential control [no $Co(terpy)_2Cl_2$], the enzyme is very active, ~1000 IU/mg, when this assay procedure is used. However, in the presence of 1 mM $Co(terpy)_2Cl_2$ (solution potential ≃ +237 mV vs. SHE) the activity measured is less than 2 IU/mg. It was also demonstrated that $Co(terpy)_2Cl_2$ did not affect the response of the electrodes to $H_2O_2$.

*Measurement of $E_{1/2}$ with Various Mediator–Titrants.* The $E_{1/2}$ of galactose oxidase was measured in the presence of several mediator–titrants to determine if they exert any specific chemical effect on $E_{1/2}$. Several mediator–titrants have been found that couple reversibly with galactose oxidase (Johnson et al., 1982a,b). Those chosen for evaluation on the basis of their compatibility with both galactose oxidase and the thin-layer cell were $K_3Fe(CN)_6$, $K_2Fe(phen)(CN)_4$, and $Co(terpy)_2Cl_2$. Data were taken by using either the oxidized or the reduced form of each of these three mediators, and the results are shown in Table I. It was also possible to measure the solution potential dependence of activity ($E_{1/2}$) without intentionally adding any mediator at all. This was done by setting a potential of +540 mV vs. SHE on the control electrode and then waiting overnight for this potential to be established in the thin layer, presumably by an extremely slow oxidation of galactose oxidase by the electrode or through trace amounts of redox active contaminants functioning as mediator–titrants. Then, after a couple of injections of 1% galactose to establish initial relative activity, the control electrode was disconnected and used to indicate solution potential within the enzyme layer. The indicated potential drifted downward (~1 mV/min), and at various times during this period injections of 1% galactose were made to determine relative activity. The relative activity data were then used to calculate ln $[(O_{ENZ}/R_{ENZ})]$ at any given indicated potential, which was then fitted to the Nernst equation with the indicated potential data to obtain $E_{1/2}$ and $n$. These data are also listed in Table I as "no mediator". It is immediately apparent that there are only subtle differences in the data obtained with each of the six mediator–titrants and with no mediator. $Fe(phen)(CN)_4^{2-}$ was chosen as the mediator–titrant to be used in the enzyme studies described below on the basis of $iR$ drop and ease of preparation considerations (Johnson, 1981).

Table I: $E_{1/2}$ and $n$ Values Obtained for Galactose at pH 7.3 Using Various Mediator–Titrants[a–c]

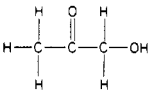
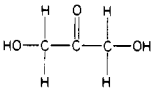
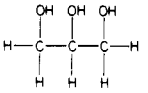
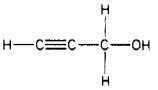
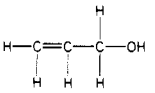
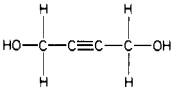
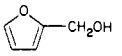
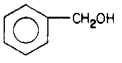
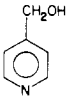
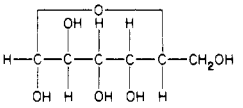
| mediator–titrant | $E^{0\prime}$ of mediator–titrant (V vs. SHE) | $\bar{E}_{1/2}$ (V vs. SHE) ± SD | average $n$ value ± SD |
|---|---|---|---|
| $Fe(CN)_6^{3-}$ | +0.457 | 0.396 ± 0.006 | 0.86 ± 0.08 |
| $Fe(CN)_6^{4-}$ | | 0.403 ± 0.005 | 0.87 ± 0.08 |
| $Fe(phen)(CN)_4^{1-}$ | +0.562 | 0.402 ± 0.004 | 0.97 ± 0.08 |
| $Fe(phen)(CN)_4^{2-}$ | | 0.403 ± 0.005 | 0.93 ± 0.08 |
| $Co(terpy)_2^{3+}$ | +0.232 | 0.406 ± 0.006 | 0.93 ± 0.04 |
| $Co(terpy)_2^{2+}$ | | 0.406 ± 0.005 | 0.98 ± 0.08 |
| no mediator[d] | | 0.402 ± 0.005 | 0.85 ± 0.20 |

[a] Galactose used as substrate; 1 mM of mediator–titrant in bulk solution (pH 7.3). [b] Data represent the average of five determinations, each done on a different membrane under enzyme-limiting conditions. [c] Average correlation coefficient for least-squares fit to the Nernst equation: 0.990–0.999. [d] Trace levels of redox active contaminants presumably establish potential in the thin layer in conjunction with the control electrode.

*Measurement of $E_{1/2}$ and n Values on Various Substrates.* The results of measuring the solution potential dependence of activity with various substrates are shown in Table II. The average correlation coefficient for the linear regression and the average maximal relative activity are also listed. In each case a substrate concentration well below the reported $K_M$ was used in the measurement. It is difficult to compare the average relative maximal activities listed in Table II with relative activity data previously reported for galactose oxidase. There are at least three problems: first, the absolute activity values which appear in the literature were obtained by a variety of assay techniques including coupled assays, which are known to activate the enzyme; second, all values reported in the literature were obtained under conditions where solution potential was not controlled or known (see Discussion); finally, the values listed in Table II are relative activities based on the steady-state response of the electrode, which prevents comparison to activities based on initial velocity measurements (see eq 2). It is apparent from these data that the solution potential dependence of galactose oxidase activity is essentially independent of which substrate is used for the measurement.

*pH Dependence of $E_{1/2}$.* $E_{1/2}$ and $n$ values were measured at several different pHs by using galactose as the substrate and 1.0 mM $Fe(phen)(CN)_4^{2-}$ as the mediator–titrant. Figure 3 shows the results of this study. At a pH of 5.5 or lower the enzyme is extremely unstable (Hamilton et al., 1978). This results in a very unstable steady state which increases the uncertainty in the measurement. It is apparent, however, that between pH 6.0 and pH 8.0 there is a dependence of $E_{1/2}$ of about 60 mV/pH unit which decreases rapidly above pH 8.0. A similar result was reported by Hamilton et al. (1978). Additionally, maximum relative activity at any given pH (not shown) was measured by setting a potential on the control electrode which was oxidizing enough to maintain all of the enzyme in the active form and then injecting a 1% galactose solution. The steady-state response obtained was then ex-

Table II: $E_{1/2}$ and $n$ Values Obtained for a Variety of Substrates[a]

| substrate ($M_r$) | structure | $\bar{E}_{1/2}$ (±0.005) (V vs. SHE) | av $n$ value (±0.1) | av correlation coefficient | av[b] maximal rel act. |
|---|---|---|---|---|---|
| acetol (74.08) |  | 0.404 | 0.87 | 0.995 | 0.07 |
| dihydroxyacetone (90.08) |  | 0.408 | 0.92 | 0.998 | 4.31 |
| glycerin (92.09) |  | 0.411 | 0.89 | 0.997 | 0.01 |
| 2-propyn-1-ol (56.06) |  | 0.411 | 0.82 | 0.998 | 0.16 |
| allyl alcohol (58.08) |  | 0.406 | 0.80 | 0.995 | 0.02 |
| 2-butyne-1,4-diol (86.02) |  | 0.406 | 0.90 | 0.998 | 0.39 |
| furfuryl alcohol (98.10) |  | 0.408 | 0.81 | 0.995 | 0.04 |
| benzyl alcohol (108.13) |  | 0.411 | 0.97 | 0.998 | 0.04 |
| 4-pyridylcarbinol (109.07) |  | 0.399 | 0.88 | 0.995 | 0.13 |
| galactose (180.16) |  | 0.403 | 0.93 | 0.998 | 1.00 |
| stachyose (666.64) | α-D-galactosyl-α-D-galactosyl-α-D-glycosyl-β-D-fructose | 0.400 | 0.89 | 0.998 | 1.44 |

[a] Enzyme membranes at 0.6 I.U./μL were used and the $E_{1/2}$ and $n$ values listed are the average of three determinations each with a different membrane. All measurements were done at pH 7.3 using 1.0 mM $Fe(phen)(CN)_4^{2-}$ as mediator-titrant. [b] Relative activity as defined in the text, but then expressed relative to the response to an equimolar concentration of galactose.

pressed relative to the response obtained at pH 7.3. The dependence of activity upon pH which was observed also agrees qualitatively below pH 8.0 with maximum velocity data taken by Hamilton's group. Above pH 8.0, however, Hamilton observed a greater dropoff in maximum velocity than our data indicate (Hamilton et al., 1978). This may be due to the fact that the enzyme in our system is stabilized with regard to high pH as a result of being cross-linked with glutaraldehyde.

## Discussion

As a result of the enzyme layer in this cell being extremely thin (0.1 μM), the solution potential can be controlled over a much broader range by using any given mediator than has been previously reported (Meckstroth, 1981). Thus, biocomponents can be studied by using mediators with $E^{0\prime}$ values as much as 300 mV away from the $E^{0\prime}$ of the biocomponent, and a wider variety of mediator–titrants can be used.

The advantages of the use of this type of cell to control solution potential with an electrode over the use of simple chemical "control" of solution potential are apparent (Johnson et al., 1982a,b). In particular, the thin-layer cell described here can be used to study $H_2O_2$-producing enzymes even in the presence of electroactive substrates and/or products. It was possible to show, for example, that acetol and di-

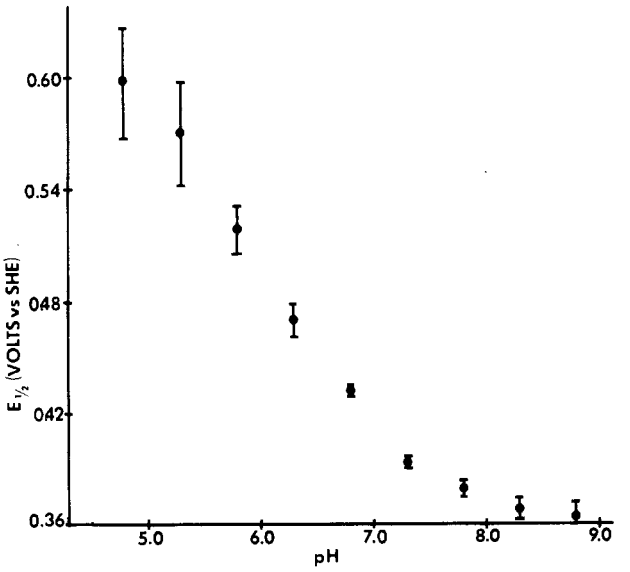



FIGURE 3: pH dependence of $E_{1/2}$ for the galactose oxidase catalyzed oxidation of galactose. The galactose concentration used was 5 mM and the mediator–titrant was 1 mM $K_2Fe(phen)(CN)_4$. Each point is the average of three determinations each made on a different membrane.

hydroxyacetone reacted with all of the mediator–titrants used in this study even though the reaction is not fast enough to significantly reduce substrate concentration on the time scale of the experiment. Without the control electrode it is impossible to maintain solution potential control so that the enzyme reaction with these substrates can be studied.

The results clearly demonstrate that it is not possible to do meaningful kinetic experiments or assays involving galactose oxidase, or any other enzyme which exhibits an activity dependence upon solution potential without solution potential control. Without such control, the apparent activity of galactose oxidase would be dependent upon both the concentration and the formal potentials of any electroactive contaminants and/or substrates present. This might be at least a partial explanation for the great range in the reported kinetic constants for galactose oxidase (Hamilton et al., 1973a).

Throughout this study, one important fact became apparent; the activity of galactose oxidase is independent both of which oxidant (or mediator–titrant) is used and of the concentration of oxidant used to activate as long as the control electrode is used to establish the solution potential of the thin layer at a constant level (Johnson et al., 1982a). This was true regardless of which substrate was used to judge activity and at oxidant concentrations much less than the stoichiometric (with enzyme) amount. In fact, as described above, it was possible to perform experiments in the absence of any added mediator–titrant. Under these conditions it is necessary to wait several hours for the control electrode to establish solution potential within the enzyme thin layer. However, after this period the steady-state $H_2O_2$ current obtained for a given concentration of any substrate is unchanged after the addition of any of the mediator–titrants as long as the control electrode remains set at a constant potential. Thus, it appears that the potential set on the control electrode alone determines the activity of galactose oxidase in this system, as long as enough time is allowed for this potential to be established in the thin layer.

At low substrate concentrations ($<K_M$) and when the steady-state current ($i_s$) is under catalysis control, the equation describing the electrode response is

$$i_s = (1/2)nFAdk_3C_EC/K_M$$

where $d$ is the thickness of the diffusion layer, $C_E$ is the concentration of immobilized enzyme in the thin layer, $C$ is the bulk substrate concentration, $k_3$ is the rate constant for the dissociation of the enzyme substrate complex into enzyme and product, $K_M$ is the Michaelis–Menten constant, and $n$, $F$, and $A$ have their usual electrochemical significance (Mell & Maloy, 1975). This equation describes the response of the electrode under all conditions described in this report. As the solution potential is varied only $k_3$, $C_E$, and/or $K_M$ could change in order to result in the change in $i_s$ which is observed here. Additional experiments will be necessary in order to distinguish among these various possibilities. Direct determinations of $k_3$ and $K_M$ in this system cannot be performed because of the influence of diffusion on the initial velocity curves (Carr & Bowers, 1980).

Several explanations for the activation of galactose oxidase by ferricyanide have been proposed. Hamilton and co-workers have suggested on the basis of electron spin resonance (ESR) and other experiments that ferricyanide and other oxidants activate the enzyme by oxidizing a catalytically inactive Cu(II) form of the enzyme to the active Cu(III) form. They propose that the catalytic mechanism involves cycling of the Cu between Cu(III) and Cu(I) states during turnover, with the Cu(III) form being the active form of the enzyme (Dyrkacz et al., 1976). The Cu(II) form occurs once every thousand or so turnovers due to the leakage of $O_2^{-}\cdot$ from the enzyme–substrate complex.

Ettinger's group, on the other hand, has presented difference absorbance and NMR data which have been interpreted to indicate that ferri- and ferrocyanide form high-affinity complexes with galactose oxidase and that this explains how ferricyanide may cause a decrease in the ESR spectra of galactose oxidase without oxidizing the Cu(II) (Bereman et al., 1977). It has also been demonstrated that catalytic amounts of horse radish peroxidase (HRP) can activate galactose oxidase (Cleveland et al., 1975). The HRP-activated enzyme has increased copper absorbance at 445 and 770 nm, similar to what is observed with activation by oxidants (Tressel et al., 1980). However, unlike oxidant-activated enzyme, the HRP-activated galactose oxidase does not exhibit reduced ESR spectral intensity. Also, oxidant activation and horse radish peroxidase activation are not synergistic, suggesting that they result in the same activated state. Ettinger's group also contends that the catalytic mechanism is sequential and has recently presented evidence that argues against the involvement of a *stable* Cu(III) form of the enzyme in the catalytic mechanism (Kwiatkowski et al., 1981).

It is significant in this study that both the activity of galactose oxidase and its dependence upon solution potential have a zero-order dependence on the concentration of the mediator–titrant and are also independent of which mediator–titrant is used. This supports the conclusion that the mediator–titrant is not directly involved in catalysis and that activation is a simple redox process. The fact that the same $E_{1/2}$ and $n$ values (within the experimental error) were obtained regardless of which substrate was used (Table II) also supports the conclusion that the mediator–titrant is not directly involved in catalysis. The data suggest that any indirect involvement of the mediator–titrant in catalysis would have to occur with a species which was common to the reaction with all the substrates or that activation occurs because the enzyme is poised in the active oxidized state before turnover begins. An interesting possibility is that oxidants activate by redox buffering an active state of the enzyme during turnover. That is, it is possible that galactose oxidase exists in active and inactive forms and that turnover is necessary to convert the inactive resting enzyme to active enzyme. Such behavior has been demonstrated in cytochrome oxidase and laccase which are both copper enzymes (Antonini et al., 1977; Hansen et al., 1984; Andreasson et al., 1976).

The dependence of $E_{1/2}$ upon pH (Figure 3) suggests that activation involves the deprotonation of a group as well as the one-electron oxidation of the same or a different group. The $pK_a$ of this group apparently lies slightly above 7.0 (Figure 3). The deprotonation of this group evidently makes the one-electron oxidation more easily accomplished.

Much of the data obtained in this study agree both qualitatively and quantitatively with the data presented by Hamilton et al., on which the proposed involvement of Cu(III) in the catalytic mechanism is partially based (Hamilton et al., 1978). The possibility that Hamilton's *kinetic* results with ferricyanide/ferrocyanide are due to specific binding to the enzyme are not consistent with the data obtained here since oxidants that are structurally and electronically quite different from ferricyanide give identical activation effects at the same potential. However, further ESR work with other oxidants will be necessary in order to settle the dispute over Cu(III). Certainly, the involvement of a particular oxidation state such as Cu(III) in the catalytic cycle cannot be confirmed or denied

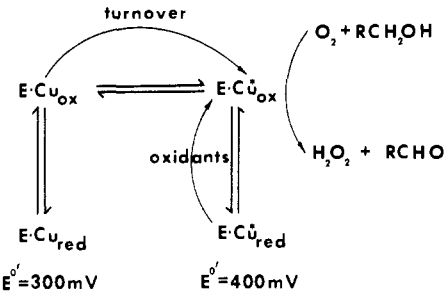



FIGURE 4: Proposed stable states of galactose oxidase. Of the four possible states only the E·Cu*$_{ox}$ state is active. Furthermore, the existance of any transient states which might occur during turnover is not addressed in this model. See text for further discussion.

on the basis of the data presented here. In fact, it is not possible to unequivocally conclude even that the enzymatic group being oxidized is the Cu atom.

The most consistent interpretation of the data, however, is that the $E^{0\prime}$ determined here is that of the enzymic Cu(II)/Cu(I). Several facts support the hypothesis that the enzyme can exist in active and inactive states. Even though, as isolated, galactose oxidase has been shown to be nearly 100% in the Cu(II) form, we speculate that this is a necessary requirement but not sufficient for galactose oxidase to be catalytically active. The fact that, in the absence of oxidants, catalytic lags are observed upon addition of substrate to solutions of galactose oxidase suggests that turnover may be autocatalytic. That is, some small portion of the enzyme might already exist in the active Cu*$_{ox}$ state (see Figure 4), and turnover by active enzyme elevates inactive Cu$_{ox}$ enzyme to active Cu*$_{ox}$ enzyme. However, as turnover continues, enzyme can still drop into the Cu*$_{red}$ state which is also inactive as indicated by the fact that galactose oxidase can be essentially shut off during turnover by adjusting the solution potential to about 240 mV vs. SHE. Oxidants, then, have their effect on steady-state catalytic activity because they maintain a high ratio of Cu*$_{ox}$ to Cu*$_{red}$ during turnover. Furthermore, we hypothesize that the Cu*$_{ox}$/Cu*$_{red}$ $E^{0\prime}$ is higher than the Cu$_{ox}$/Cu$_{red}$ $E^{0\prime}$. The discrepancy in the $E^{0\prime}$ determined here and that determined by Ettinger ($E^{0\prime}$ = +0.300 V vs. SHE) on the resting enzyme by two different techniques could be explained by such a model (Kosman & Ettinger, 1981). Alternatively, as mentioned above, many of the facts are also consistent with the active form of the enzyme involving Cu(III). However, Ettinger has shown through absorbance measurements that the $E^{0\prime}$ he measures on the resting enzyme is that of the Cu(II)/Cu(I) transition, and no one has observed more than one one-electron transition in galactose oxidase in the positive potential range. Therefore, the simplest interpretation is that the $E^{0\prime}$ obtained here is that of the active-state enzymic Cu(II)/Cu(I). Another possibility which cannot be excluded is that the disagreement with Ettinger's result could be otherwise related to the fact that he did his measurements under anaerobic conditions on resting enzyme whereas our results and Hamilton's were obtained during turnover. For example, whether or not $O_2$ is necessary in combination with oxidant for activation either before or after turnover begins has not been addressed. Further experiments will be necessary in order to resolve this discrepancy.

In conclusion, we have described the determination of the solution potential dependence of activity for galactose oxidase. The $E_{1/2}$ and $n$ values obtained are +0.40 ± 0.005 V vs. SHE and 0.9 ± 0.1 electron at pH 7.3. There are several possible interpretations of our data; however, we believe the one that best fits and is most consistent with the work of Hamilton and Ettinger is that the $E_{1/2}$ we are measuring is the $E^{0\prime}$ of the enzymic Cu(II)/Cu(I) in the active state of the enzyme. Furthermore, we propose that the enzymic Cu(II)/Cu(I) $E^{0\prime}$ is higher in the active form of the enzyme than in the inactive form and turnover is required to convert inactive to active enzyme. Oxidants are essential activators and activate because they redox buffer the active state of the enzyme during turnover; that is, they maintain a higher enzymic Cu(II):Cu(I) ratio. More experiments will be necessary in order to prove or disprove this model. Finally, the pH dependence of $E_{1/2}$ suggests that deprotonation of an enzymic group facilitates activation. Because of the nature of this redox activation we believe that it is probably important, in vivo, in modulating galactose oxidase activity. Moreover, it is also likely that other enzyme systems exist in nature in which activity is regulated by solution potential. For example, preliminary results using xanthine oxidase and diamine oxidase in this system demonstrate that solution potential affects the activity of these enzymes as well.

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**Registry No.** $Fe(CN)_6^{3-}$, 13408-62-3; $Fe(CN)_6^{4-}$, 13408-63-4; $Fe(phen)(CN)_4^{1-}$, 65749-14-6; $Fe(phen)(CN)_4^{2-}$, 17455-55-9; $Co(terpy)_2^{3+}$, 18308-16-2; $Co(terpy)_2^{2+}$, 19137-07-6; acetol, 116-09-6; dihydroxyacetone, 96-26-4; glycerin, 56-81-5; 2-propyn-1-ol, 107-19-7; allyl alcohol, 107-18-6; 2-butyne-1,4-diol, 110-65-6; furfuryl alcohol, 98-00-0; benzyl alcohol, 100-51-6; 4-pyridylcarbinol, 586-95-8; galactose, 59-23-4; stachyose, 470-55-3; galactose oxidase, 9028-79-9.

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# Kinetics of Conformational Changes and Inactivation of Human $\alpha_2$-Macroglobulin on Reaction with Methylamine†

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ABSTRACT: Previous studies have shown that the thio ester bonds of human $\alpha_2$-macroglobulin ($\alpha_2$M), of which one is located in each of the four subunits of the protein, are cleaved independently and equivalently by small primary amines in a reaction occurring under pseudo-first-order conditions [Larsson, L.-J., & Björk, I. (1984) *Biochemistry 23*, 2802–2807]. In this work, the kinetics of the conformational change that accompanies cleavage of these bonds have been characterized with the most reactive amine, methylamine, by measurements of the changes of the spectroscopic and hydrodynamic properties of the protein and of the decrease of its proteinase binding capacity. These changes occurred on a longer time scale than the thio ester bond cleavage and, in contrast to this cleavage, showed sigmoidal kinetics. This behavior suggests that the conformational change is subsequent to thio ester cleavage. Moreover, the change of the tryptophan fluorescence of the protein preceded the changes of the ultraviolet absorbance, hydrodynamic volume, and activity, indicating that the conformational change involves at least two successive steps. Nonlinear least-squares fits of the rate of change of tryptophan fluorescence to different models suggested that an initial, limited conformational change occurs in each $\alpha_2$M half-molecule when both thio ester bonds of the dimeric unit have been cleaved by the amine. The rate constant of this change is considerably higher than the pseudo-first-order rate constant for the thio ester bond cleavage at the methylamine concentrations investigated. These findings are consistent with previous evidence suggesting that the half-molecule is the functional unit of $\alpha_2$M. The kinetics of the change of ultraviolet absorbance, which occurred concurrently with the decrease of hydrodynamic volume and activity of the protein, could be reasonably well fitted to models involving either two sequential conformational changes within the half-molecule or a cooperative conformational change in the whole $\alpha_2$M tetramer occurring after the initial changes in both half-molecules have been completed. However, alternative mechanisms, in particular more than two successive conformational steps, cannot be excluded.

$\alpha_2$-Macroglobulin ($\alpha_2$M)[1] is a high molecular weight ($M_r$ ~725 000) plasma proteinase inhibitor consisting of four identical subunits (Jones et al., 1972; Hall & Roberts, 1978). It is unique as a proteinase inhibitor in that it inactivates a wide variety of proteinases from different classes and with different specificities (Barrett & Starkey, 1973; Harpel, 1976). The initial step in the binding of the proteinase is a proteolytic cleavage by the enzyme of a limited region of the polypeptide chain of $\alpha_2$M, the "bait" region (Harpel, 1973; Barrett et al., 1979; Swenson & Howard, 1979a; Sottrup-Jensen et al., 1981b). This cleavage induces a conformational change of the inhibitor (Barrett et al., 1974, 1979; Björk & Fish, 1982; Gonias et al., 1982; Branegård et al., 1982) that leads to a noncovalent binding ("entrapment") of the proteinase (Barrett & Starkey, 1973). Most evidence indicates that the maximal stoichiometry of binding of enzyme to inhibitor is 2:1 (Ganrot, 1966; Barrett et al., 1979; Swenson & Howard, 1979a; Sottrup-Jensen et al., 1980; Pochon et al., 1981; Björk et al., 1984). However, under certain conditions, some binding sites

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[1] Abbreviations: $\alpha_2$M, $\alpha_2$-macroglobulin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; STI, soybean trypsin inhibitor; EDTA, ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane.

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