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FLUORIDE ION AS AN NMR RELAXATION PROBE OF GALACTOSE OXIDASE—SUBSTRATE BINDING *

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Summary

From the dependence on substrate concentration of fluoride ion spin-lattice and spin-spin paramagnetic relaxation rate enhancements, a value for the dissociation constant, $K_d = 0.059 \pm 0.002$ M, for the anaerobic binding of dihydroxyacetone (monomer) to the Cu(II) site of the enzyme galactose oxidase (D-galactose:oxygen 6-oxidoreductase, EC 1.1.3.9) has been obtained. This value for K_d lies between previously reported values for K_m derived by use of classical Michaelis-Menten kinetics. An analogous calculation for the anaerobic binding of galactose to the enzyme yields $K_d = 0.145 \pm 0.004$ M, a value different from several reported Michaelis constants. F^- NMR relaxation measurements on air-exposed samples of galactose and the enzyme yield a dissociation constant for the active site-oxidation product (presumed to be galactohexodialdose), $K_d = 2.2 \pm 0.2$ M, a value at least an order of magnitude larger than the Michaelis or dissociation constants calculated for the binding of galactose to the enzyme active site; no value for this constant had been reported previously. Some implications of the competition results for the type of substrate binding are discussed.

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Abbreviations used: $1/T_1$, spin-lattice relaxation rate; $1/T_2$, spin-spin relaxation rate; $1/T_{1p}$, $1/T_{2p}$, paramagnetic spin-lattice and spin-spin relaxation rates.

Introduction

Galactose oxidase (D-galactose: oxygen 6-oxidoreductase, EC 1.1.3.9) catalyzes the conversion of many primary alcohols to the corresponding aldehydes with the concomitant reduction of O_2 to H_2O_2 [1—4]. Galactose oxidase is of particular interest in that it may be the only enzyme which contains just a single Cu(II) per enzyme molecule and no other metal atoms [3,5]. Recent studies [6—8] suggest that alcohol substrates bind at an outer sphere or axial coordination site, relative to the Cu(II) ion at the galactose oxidase active center and that exogenous ligands (such as F^- or CN^-) bind to the Cu(II) at an equatorial position. Fluoride also binds more weakly to the galactose oxidase Cu(II) at an axial coordination site (Marwedel et al., unpublished data and Ref. 9); it is this weakly bound F^- (K_d approx. 50 M [9]) which gives rise to the ^{19}F-NMR relaxation enhancement produced by galactose oxidase.

The specificity of galactose oxidase for D-galactose, compared to other hexoses, is evident from enzyme activity studies [10]. The 4-position of galactose appears to be critical in that glucose or 4-*O*-methylgalactose, which differ from galactose at this position, do not act as substrates for galactose oxidase [10]. Moreover, 4-deoxygalactose is a poor substrate [11], whereas 4-fluorogalactose and 4-aminogalactose are somewhat better [12]. These results have been interpreted to indicate that substituents at the substrate 4-position may form hydrogen bonds to the protein [11].

Michaelis constants for galactose and dihydroxyacetone (an excellent substrate), as reported in previous studies [2,6,11,13,14], are listed in Table I. The reported K_m values for galactose vary by almost a factor of 10^2 and those for dihydroxyacetone by almost a factor of 10. Moreover, these Michaelis constants, even if correctly determined, may differ from the equilibrium dissociation constant value for a given substrate, since the mechanism for the galactose, O_2 reaction has been shown to be a sequential, bisubstrate-ordered one, for which the rapid equilibrium condition does not hold [6]. Also, the binding of the aldehyde oxidation products of galactose and dihydroxyacetone

TABLE I

MICHAELIS CONSTANTS OF GALACTOSE OXIDASE SUBSTRATES

Fixed substrate, O_2, at presumably non-saturating concentration (corresponding to atmospheric pressure of O_2, or less, above the solution, cf. Ref. 6).

Variable substrate	K_m (M)	Assay used	Reference
Galactose	0.24	peroxidase-chromogen	13
	0.175	O_2 uptake	6
	0.038	O_2 uptake	2
	0.0295	peroxidase-chromogen	11
	0.008	O_2 uptake	11
	0.0028	peroxidase-chromogen	14
Dihydroxyacetone (monomer)	0.11	peroxidase-chromogen	11
	0.022	O_2 uptake	2
	0.0121	O_2 uptake	11

has not, to our knowledge, been analyzed successfully by use of Michaelis-Menten kinetics.

In order to assess more quantitatively the extent of substrate and product binding to galactose oxidase for galactose and dihydroxyacetone, we have carried out ^{19}F-NMR relaxation studies using F^- as a probe of the Cu(II) center. Preliminary results [15,16] had indicated that values for substrate and oxidation product dissociation constants could be obtained from the changes in ^{19}F-NMR relaxation rates, produced by added substrate under appropriate aerobic or anaerobic conditions. Another aspect of such a study was to gain supporting evidence for the conclusion that substrate was bound to galactose oxidase at an outer sphere or axial position relative to the Cu(II) and that F^- detected by ^{19}F-NMR relaxation effects coordinated axially to the Cu(II) ion; these inferences were drawn from the results of kinetics [6,7], ^{13}C-NMR [7] and ^{19}F-NMR relaxation rate (Marwedel et al., unpublished data and Ref. 9) investigations.

Water proton-NMR relaxation data cannot be used as readily as the fluoride ^{19}F-NMR data to probe substrate binding in this system (as we demonstrate below and has been shown elsewhere [9,15,16,23]) because the contribution of nonspecific and/or outer-sphere effects to the proton relaxation rates may constitute a relatively large part of the total. The effect of binding on substrate ^{13}C- or ^{1}H-NMR lines is also too small to be useful for determination of dissociation constants [7]. Thus, the F^- relaxation experiments afford a relatively convenient and reliable method to supplement kinetic studies of substrate-enzyme equilibria, where, in particular, kinetic data are not available (as for the binding of the aldehyde oxidation product of galactose to galactose oxidase) or where other spectral effects due to substrate-enzyme complexation are small.

Experimental

Aqueous F^- solutions were stored or transferred in polyethylene or Kel-F equipment. Previous investigations had shown that glass and polyvinylchloride cause irreversible changes in aqueous fluoride solutions [17,18]. In preliminary NMR studies with aqueous F^- contained in glass sample tubes, both $1/T_1$ and $1/T_2$ were 1—3-times greater for samples in a glass tube compared to those rates for samples of the same stock solution in a Kel-F tube. In the presence of 0.10 mM galactose oxidase, observed F^- relaxation rates are identical in glass, Delrin and Kel-F sample tubes over periods of several hours after sample preparations. However, over the 24 h time course required for some of the NMR measurements both glass and Delrin produce alterations in the solutions as indicated by an increase in the aqueous F^- relaxation rates measured in the presence and absence of galactose oxidase. Relaxation rates obtained for analogous solutions in Kel-F sample tubes were reproducible over a 3-day period.

All relaxation rates reported here were measured in sample tubes machined from 7 mm o.d. Kel-F rods. The buffer solutions used in the NMR experiments were made using deionized, glass-distilled H_2O which contained less than 10^{-8} M Cu(II) ion, as determined by copper atomic absorption (limit of detection =

10^{-8} M Cu(II). Buffers used to prepare stock solutions were passed through a Chelex-100 (Bio-Rad Laboratories) column to remove any Cu(II) ion impurity present in the phosphate and NaOH components. The galactose oxidase used in the NMR experiments was exhaustively dialyzed against Cu(II)-free buffers to which 10^{-7} M ethylenediaminetetraacetic acid was added to insure the complexation of any free Cu(II) ion present due to enzyme decomposition. The stock solutions and NMR samples, used in the water proton and F^- relaxation measurements, were maintained at pH = 7.0 ± 0.10 by use of 0.05 M NaH_2PO_4/NaOH. The ionic strength of all F^--NMR samples was maintained at 1.0 ± 0.1 M by the addition of K_2SO_4. Increasing ionic strength had been observed not only to enhance enzyme activity [3], but also to increase aqueous F^- relaxation rates [16,17].

Galactose oxidase was purified in the laboratory of Dr. Daniel J. Kosman from the extracellular media of cultures of the fungus *Dactylium dendroides* by use of modified literature procedures [19]. Protein concentrations were calculated from the 280 nm absorbance of solutions by use of an assumed molar extinction coefficient of 105 000 $M^{-1} \cdot cm^{-1}$ [19]. Enzyme activity was determined by means of a standard coupled assay reaction monitored at 460 nm [19].

Galactose, glucose and dihydroxyacetone were dried under vacuum and stored under dry argon or nitrogen. In the aerobic experiments, glucose was weighed into a sample tube containing the appropriate F^- or galactose oxidase, F^- solution. Aerobic glucose samples were also prepared from volumetrically calibrated F^-, glucose solutions; the relaxation rates measured for such samples agreed well with those for glucose samples prepared directly in the NMR tube. Anaerobic additions of glucose, galactose or dihydroxyacetone were effected by weighing the solid into a sample tube containing the appropriate deoxygenated solution. The deoxygenated solutions were prepared by a prolonged bubbling of wet nitrogen or argon through the solution, prior to transfer into the sample tube. Since denaturation was sometimes observed after prolonged, vigorous bubbling, assays and 280 nm absorbance readings for aliquots of filtered galactose oxidase samples before and after deoxygenation were used to determine enzyme viability. Deoxygenation and transfer of solutions and solids were carried out under nitrogen or argon in a glove bag. Tightly fitting polyethylene caps and Parafilm effectively sealed the NMR tube during the course of an experiment. However, in some samples oxygen leakage was detected by relaxation rate changes observed after the required sample equilibration period (1—3 h); dilute aliquots of the few samples which showed such effects sometimes exhibited a significantly smaller specific activity. Conversion of several anaerobic samples to aerobic was carried out by slow passage of wet O_2 over the surface of the deoxygenated sample for 10—15 min.

Slight, but measurable volume changes (increases of 5—10%) were observed on addition of solids (glucose, dihydroxyacetone, galactose) to aqueous solutions containing F^-; similar volume increments were observed on addition of glucose and dihydroxyacetone to anaerobic solutions containing galactose oxidase and F^-. Much larger volume expansions (10—20%) were observed on the addition of galactose to anaerobic solutions of galactose oxidase and F^-. Measured volumes in marked sample tubes and changes in the 280 nm absor-

bance were used to estimate these volume changes; for dihydroxyacetone, only the former method was used since this compound has a substantial absorbance at 280 nm.

^{19}F- and ^{1}H-pulsed NMR relaxation rate measurements at 56.4 MHz (^{19}F) or 60 MHz (^{1}H) were taken on a Bruker-BK/R-321s variable frequency spectrometer. Field-frequency control was maintained via an external lock sample of paramagnetically doped water. Relaxation rate measurements were obtained for non-spinning samples by means of the Carr-Purcell Meiboom-Gill pulse sequences [20,21] or Carr-Purcell (180°—τ—90°) pulse sequences [21]. Relaxation rates were obtained from the slopes calculated from a linear least-squares fit of appropriate plots. Error limits, when given, correspond to the standard deviation in the slope derived from the method. In the computer program used, the data were subjected to the criteria of a standard Students' t-test.

Results

From the data presented in Table II, it is evident that the relaxation rate enhancements due to the holoenzyme are 40—50-times larger for fluoride (1.0 M) than for water protons. A comparison of the relaxation rates observed in the presence of the holoenzyme with those due to the apoenzyme shows that the nonspecific binding of F^- to galactose oxidase provides a contribution to the observed relaxation rate enhancements that is within experimental error (±10%). The non-specific binding of water to enzyme, however, provides a major contribution (30—100%) to the observed relaxation rate enhancements of water protons.

The paramagnetic relaxation rates due to the Cu(II) center in galactose oxidase, $1/T_{1p}$ and $1/T_{2p}$, are calculated as the difference between the relaxation rates observed in the presence of the holoenzyme and those observed for

TABLE II

F^- AND WATER PROTON RELAXATION RATE ENHANCEMENTS IN THE PRESENCE OF GALACTOSE OXIDASE

$1/T_1$, $1/T_2$ are relaxation rates measured in the presence of galactose oxidase; $1/T'_{1A}$, $1/T'_{2A}$ are relaxation rates observed in aqueous solution. All samples were prepared in 0.05 M, pH 7.0 phosphate buffer; ionic strength was maintained at 1.0 M by use of K_2SO_4. Temperature was 27 ± 2°C. n.m., not measured.

Galactose oxidase ($M \cdot 10^4$)	F^- (M)	$(1/T_1 - 1/T'_{1A})$ (s^{-1})		$(1/T_2 - 1/T'_{2A})$ (s^{-1})	
		holo	apo	holo	apo
F^- relaxation					
1.5 ± 0.10	1.0 ± 0.05	21.3	2.49	53.1	4.72
1.1 ± 0.10	1.0 ± 0.05	15.1	0.703	39.8	1.31
0.83 ± 0.05	1.0 ± 0.05	10.7	0.434	29.8	0.844
0.68 ± 0.05	1.0 ± 0.05	8.43	0.226	24.2	0.693
0.32 ± 0.03	1.0 ± 0.05	3.98	n.m.	10.6	n.m.
Water proton relaxation					
1.2 ± 0.10	none	0.378	n.m.	0.935	n.m.
0.80 ± 0.06	none	0.240	n.m.	0.705	n.m.
0.32 ± 0.05	none	0.094	0.107	0.311	0.110
0.29 ± 0.03	1.0 ± 0.02	0.051	n.m.	0.271	n.m.

an analogous sample containing apoenzyme. There is no discernible contribution to $1/T_{1p}$ and $1/T_{2p}$ from dissolved oxygen; F^- and water proton relaxation rates obtained under aerobic and anaerobic conditions in the presence of galactose oxidase are the same within experimental error. Although it is not evident from the data in Table II, the variation of paramagnetic relaxation rates with galactose oxidase concentration is somewhat non-linear for concentrations

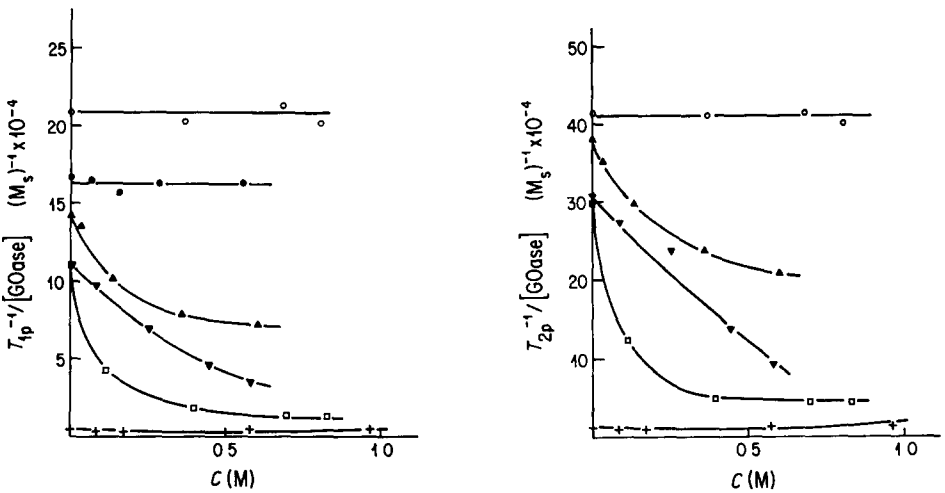

Fig. 1. F^- spin-lattice relaxation rate enhancements observed in the presence of galactose oxidase (GOase) and various amounts of glucose, galactose, or dihydroxyacetone. Samples were prepared in 0.05 M, pH 7.0 phosphate buffer. Measurements were taken at 26 ± 1.5°C on the following samples (numbers in parentheses represent estimated errors):

Galactose oxidase $M \cdot 10^4$	F^- (M)	Glucose (M)	Dihydroxy acetone (M)	Galactose (M)	Anerobic conditions?
1.12 (0.05)	0.82 (0.05)	●			no
1.29 (0.10)	0.94 (0.06)	○			yes
0.85 (0.05)	1.00 (0.03)		□		yes
1.11 (0.10)	0.89 (0.10)			▲	yes
0.67 (0.06)	0.76 (0.06)			▼	no
2.00 (0.07)	H_2O			+	yes

Fig. 2. F^- spin-spin relaxation rate enhancements observed in the presence of galactose oxidase (GOase) and various amounts of glucose, galactose or dihydroxyacetone. Samples were prepared in 0.05 M, pH 7.0 phosphate buffer. Measurements were taken at 26 ± 1.5°C on the following samples (numbers in parentheses represent estimated errors)·

Galactose oxidase $M \cdot 10^4$	F^- (M)	Glucose (M)	Dihydroxy acetone (M)	Galactose (M)	Anerobic conditions?
1.29 (0.10)	0.94 (0.06)	○			yes
0.85 (0.05)	1.00 (0.03)		□		yes
1.11 (0.10)	0.89 (0.10)			▲	yes
0.67 (0.06)	0.76 (0.06)			▼	no
2.00 (0.07)	H_2O			+	yes

greater than about 10^{-4} M [15]. Nevertheless, in order to magnify the effects of substrate competition with F^-, galactose oxidase concentrations in the range $0.67 \cdot 10^{-4}$—$1.29 \cdot 10^{-4}$ M were used (see Figs. 1 and 2).

Figs. 1 and 2 depict F^- paramagnetic relaxation rates due to the Cu(II) center in galactose oxidase, measured for solutions containing varying amounts of D-glucose, D-galactose and monomeric dihydroxyacetone. It is evident that D-glucose does not bind appreciably to galactose oxidase Cu(II) either in the presence or absence of O_2. This result is in accord with several previous investigations [2]. Dihydroxyacetone has been shown to be an excellent substrate for galactose oxidase [2,11]; its presence has a greater de-enhancement effect on the observed fluoride relaxation rates than that of D-galactose. A comparison of the observed F^- paramagnetic relaxation rate de-enhancements due to D-galactose in the presence of O_2 with those measured in the absence of O_2 shows that the products, D-galactohexodialdose and hydrogen peroxide, have a distinctly different de-enhancement effect on fluoride relaxation rates than do the substrates, galactose and O_2. *

We assume that the initial de-enhancements observed in Figs. 1 and 2 are due to either of the following: (a) F^- bound to the enzyme Cu(II) is released as the alcohol binds; (b) the alcohol binds to enzyme Cu(II) and prevents the F^- from exchanging with bulk fluoride. Both alternatives correspond to observed de-enhancements due to the 'removal' of exchangeable F^-: one bound substrate molecule effectively removes an NMR detectable F^-.

The removal scheme can be described by the following equation:

$$f(T_{ip}) = ((1/T_{ip})_n^0 - (1/T_{ip})_n)^{-1} = \frac{K_d(1 + K_F C_F)^2\, T_{iM}}{K_F} \cdot \frac{1}{C} + \frac{(1 + K_F C_F)\, T_{iM}}{K_F} \tag{1}$$

where $i = 1,2$; the subscript, n, denotes normalized paramagnetic relaxation rates; the superscript, 0, designates relaxation rates observed in the absence of substrate (or product); C is the total concentration of the substrate (or product); C_F is the total concentration of fluoride; K_d is the substrate (or product) dissociation constant in units of M; K_F is the F^- binding constant in units of M^{-1}; T_{1M} and T_{2M} are the intrinsic paramagnetic relaxation times at the bound site.

If our assumptions are correct, a plot of $f(T_{ip})$ ($i = 1,2$) vs. $1/C$ should yield a straight line characterized by the following parameters:

$$m = \text{slope} = \frac{K_d(1 + K_F C_F)^2\, T_{iM}}{K_F}, \tag{2a}$$

$$B = f(T_{ip}) - \text{intercept} = \frac{(1 + K_F C_F)\, T_{iM}}{K_F}, \tag{2b}$$

$$1/K_{app} = m/B = K_d(1 + K_F C_F) = -(1/C - \text{intercept}) \tag{2c}$$

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* In Figs. 1 and 2 the normalized relaxation rates, $(1/T_{ip})_n \equiv (1/T_{ip}) \times 1/[\text{galactose oxidase}]$, $i = 1$ or 2, do not have precisely the same values for different galactose oxidase concentrations; as mentioned in the text, variation of relaxation rate with galactose oxidase concentration is somewhat non-linear in the range employed. This non-linear variation presumably reflects the dependence of solution viscosity on [galactose oxidase] at the high enzyme concentration used [16].

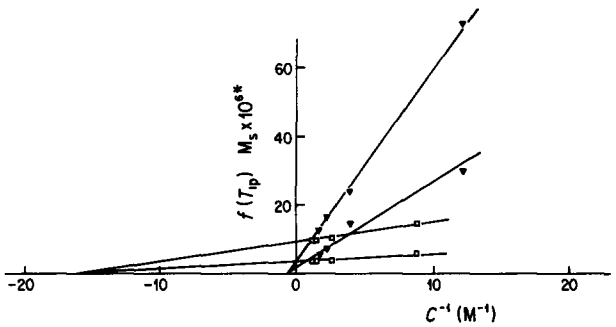

Fig. 3. Calculation of apparent substrate (or product) dissociation constants for galactose product (▼) and dihydroxyacetone (□) binding to galactose oxidase by use of the relaxation rate data displayed in Figs. 1 and 2 and Eqn. 1. See Figs. 1 and 2 for experimental conditions; upper curve of the pair for each corresponding set of points (▼ or □) is from spin-lattice (T_1) data, lower for spin-spin (T_2).

Several examples of these double-reciprocal plots are displayed in Fig. 3. The parameters obtained from Eqn. 2a—c and the plots are presented in Table III.

The validity of the assumptions used for the derivation of Eqn. 1 can easily be tested. Our model implies that the binding of substrate (or product) does not change the intrinsic relaxation rates of enzyme Cu(II) bound fluoride, $1/T^0_{1M}$ and $1/T^0_{2M}$. If the assumptions used in the derivation of Eqn. 1 are correct, the ratios of the intrinsic fluoride relaxation rates in the presence of the substrate (or product), $r_{2,1}$, should equal the ratio in the absence of substrate (or product), $r^0_{2,1} = T^0_{1M}/T^0_{2M}$. Inspection of Eqn. 2a—b shows that the ratio $r_{2,1}$ is given by

$$\frac{m(T_{1p})}{m(T_{2p})} = \frac{B(T_{1p})}{B(T_{2p})} = \frac{T_{1M}}{T_{2M}} = r_{2,1} = r^0_{2,1} = \frac{(1/T_{2p})^0_n}{(1/T_{1p})^0_n}. \quad (3)$$

By use of Eqn. 3, we find that $r^0_{2,1}$ varies from 2.4 to 2.8 for samples containing the high concentrations of galactose oxidase used in these experiments

TABLE III

LIGAND BINDING TO GALACTOSE OXIDASE

Parameters were calculated from linear plots such as these displayed in Fig. 3 (see text). The symbols correspond to those in the legends of Figs. 1 and 2 where the conditions of each experiment are described. The K_{app} is calculated from the intercepts of plots such as those displayed in Fig. 3. Numbers in parentheses are calculated by use of Eq. 2c· $K_{app} = B/m$.

Experiment and symbol	Plot	m ($M_s^2 \cdot 10^6$)	B ($M_s \cdot 10^6$)	K_{app} (M^{-1})
Aerobic				
F^-, galactose ▼	$f(T_{1p})$	5.54	2.6	0.45 (0.47)
	$f(T_{2p})$	2.56	1.0	0.45 (0.39)
Anaerobic				
F^-, galactose ▲	$f(T_{1p})$	1.69	11.5	6.6 (6.8)
	$f(T_{2p})$	0.82	5.8	6.6 (7.1)
F^-, dihydroxyacetone □	$f(T_{1p})$	0.56	9.4	15.9 (16.8)
	$f(T_{2p})$	0.20	3.4	16.2 (17.0)

(see Table II). The values of $r_{2,1}$ in the aerobic F^-, galactose experiments (▼) and the anaerobic F^-, dihydroxyacetone experiments (□) are 2.6 and 2.8, respectively, in good agreement with observed values of $r^0_{2,1}$; Eqn. 1 appears to be valid for these experiments. The calculated values of $r_{2,1}$ for the anaerobic F^-, galactose experiments (▲) (see Figs. 1 and 2) are 2.0; the deviation of this value from observed values of $r^0_{2,1}$ suggests that Eqn. 1 does not completely describe the effects of galactose on F^- relaxation rate enhancements due to enzyme Cu(II). That the effects of galactose binding to the active site are different from those due to the binding of dihydroxyacetone (or the oxidation products) is also indicated by an observed relaxation rate enhancement seen for both F^- and water protons in the presence of high concentrations of galactose and galactose oxidase [16].

If we assume that $K_F = 0.022\ M^{-1}$ [9], we can calculate a dissociation constant for the active site-dihydroxyacetone complex by use of Eqn. 2d:

$$K_d = \frac{1}{K_{app}(1 + K_F C_F)} \tag{2d}$$

and the K_{app} values given in Table III. We find that $K_d = 0.059 \pm 0.002$ M which lies between K_m values (see Table I) reported previously. Similarly, we can calculate a dissociation constant for the active site-oxidation product complex: $K_d = 2.2 \pm 0.2$ M. A similar calculation of the dissociation constant for the anaerobic binding of galactose to the active site gives: $K_d = 0.145 \pm 0.004$ M, a value which is an order of magnitude larger (see Table I) than some K_m values previously reported [2,11,14], but which agrees reasonably well with a recent determination [6]. Attempts to fit the anaerobic F^-, galactose ^{19}F-NMR relaxation data to more elaborate relaxation or binding schemes have been unsuccessful. Possibly the discrepancy in the ratio $r_{2,1}$ is due to a de-enhancement effect caused by the 'removal' of exchangeable bound fluoride combined with an enhancement effect caused by the presence of additional exchangeable F^- hydrogen-bonded to the -OH moieties of galactose molecules near the galactose oxidase Cu(II) center. Another explanation might be that bound galactose makes F^- equatorially coordinated to the Cu(II) more labile, so that it contributes to the observed ^{19}F relaxation (see Discussion).

Discussion

The much greater relaxation rates observed for axially bound fluoride compared to those for water protons demonstrate the utility of F^- as a probe for paramagnetic metalloenzymes. The dipolar interaction between unpaired electron spin density in p-orbitals centered on the fluorine nucleus and the ^{19}F nucleus is a dominant factor in the ^{19}F relaxation mechanism (Marwedel et al., unpublished data and Refs 9, 17). A small fraction of unpaired spin density present in the fluorine p-orbital gives rise to a much greater relaxation effect than that due to the greater unpaired spin density on the metal ion since the average distance of the unpaired spin to the ^{19}F nucleus is much smaller in the fluorine p-orbital than in metal centered orbitals [22].

The competitive scheme used to interpret the relaxation effects of added glucose, galactose and dihydroxyacetone assumes that the binding of one

substrate (or product) molecule to enzyme Cu(II) 'removes' an axially-coordinated, NMR-detectable F^-. The model ignores the equatorial fluoride which is assumed to exchange too slowly with F^- in solution to give any ^{19}F-NMR relaxation effects (Marwedel et al., unpublished data and Ref. 9). Conceivably, binding of galactose to enzyme Cu(II) in an axial position could decrease the exchange life-time of the equatorially-coordinated fluoride and, thus increase the observed fluoride relaxation rates. NMR measurements on F^-, galactose and F^-, glucose solutions indicate, however, that fluoride coordinates to alcohol molecules, presumably via hydrogen bonds to the hydroxyl group protons. For this reason, we believe that the galactose data do not completely fit the proposed competition scheme described by Eqn. 1 because of additional fluoride bound to the galactose molecules at the active site.

In any case, it is evident that galactose (and its oxidation products) and dihydroxyacetone effectively compete with Cu(II) bound fluoride while glucose does not. The calculated dissociation constants for dihydroxyacetone and galactose agree reasonably well with some, if not all values, reported for Michaelis constants and the consistency test (that $r_{2,1} \cong r^0_{2,1}$) shows that the anaerobic binding of dihydroxyacetone to the Cu(II) fits the model used here for analysis of relaxation rate changes. Since the analysis of aerobic binding of galactose to galactose oxidase also satisfies this consistency requirement, we believe that the dissociation constant calculated for the oxidation product is quite reliable.

Our assumption that the oxidation product which affects the ^{19}F-NMR relaxation rates is galactohexodialdose and not hydrogen peroxide, is justified as follows. Clearly the oxidation product binds very weakly to the Cu(II), at least at the high salt concentrations used in the NMR experiments ($K_d \sim 2M$). On the other hand H_2O_2 binds much more strongly to the enzyme than the K_d value derived here would indicate (K_m approx. 0.010 M for H_2O_2 [6]). Thus if the binding of H_2O_2 did interfere with the axial binding of F^- in the aerobic experiments with galactose, one would expect a K_d value about 100-times smaller than that derived in this work. Moreover, it is likely that enzyme bound H_2O_2 (possibly equatorially coordinated to the Cu(II)) does not alter appreciably the intrinsic relaxation rates of axially coordinated F^-, since the consistency test ($r_{2,1} \cong r^0_{2,1}$) is satisfied.

It is of interest that in the absence of an alcohol substrate, the presence of paramagnetic O_2 has no apparent effect on observed fluoride or water proton relaxation rates due to enzyme Cu(II). This result suggests that one (or both) of the following two situations obtains: (a) O_2 binds weakly to the Cu(II) ion in the absence of substrate, (b) O_2 binds to the active site relatively far away from the axially-coordinated fluoride. Kinetic studies [6] are consistent with the former conclusion.

Our results are consistent with coordination models in which substrate binds either axially to the galactose oxidase Cu(II) or at an outer sphere site near the metal. If the binding site was far removed from the copper, then it would be necessary that bound substrate bring about a galactose oxidase conformational change that would prevent axially bound F^- from exchanging with F^- in bulk solution; such a conclusion is not warranted, if one argues either from an Okham's Razor type principle, i.e., that the simplest consistent explanation is

the preferred one, or from the results of competition between F^- and the products of galactose oxidation (vide supra). Either axial or outer sphere substrate coordination would allow for direct electron transfer between galactose oxidase Cu(II) and substrate, as has been proposed recently [4], although neither requires that such direct electron transfer necessarily occurs as part of the catalytic mechanism. Additional F^- relaxation rate studies on tryptophan modified [3] and histidine-alkylated galactose oxidase [9] may be used in defining more clearly the role of these active site residues and the nature of substrate binding.

It is evident from our results that F^--NMR relaxation data can provide both quantitative and qualitative information about substrate binding to type 2 Cu(II) centers in metalloenzymes, such as that in galactose oxidase. The use of F^- as an NMR relaxation probe may be particularly valuable for investigating those substrate or product species whose binding cannot be readily analyzed from conventional kinetic studies or which form enzyme-substrate complexes too weak to yield significant spectroscopic effects. Fluoride may also be useful for those cases where water proton relaxation enhancements are small or complicated by the presence of appreciable outersphere effects, as in the case for galactose oxidase, and as for a recently reported ^{19}F-NMR study of superoxide dismutase [24].

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