FLUORIDE ION AS A NMR RELAXATION PROBE OF PARAMAGNETIC METALLOENZYMES: THE BINDING OF FLUORIDE TO GALACTOSE OXIDASE[1]

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Summary: The use of fluoride ion, as a novel nuclear magnetic resonance relaxation probe of paramagnetic metalloenzymes, has been tested on galactose oxidase. The concentration dependence of the F^- longitudinal relaxation rates, $R_1 = 1/T_1$, and competition studies with CN^- demonstrate that F^- binds to the enzyme at or near the Cu^{+2} site with a binding constant of the order of 1 M^{-1}. Competition studies with galactose indicate that a ternary or higher order complex between enzyme, galactose and F^- is formed.

Introduction: In a preliminary study of water proton relaxation as a probe of the Cu^{+2} site in galactose oxidase (GOase[2]), we found relatively small relaxation enhancements at the maximum concentration of GOase that could be employed and no significant effects due to substrate or O_2 (see Table I). These results could be rationalized on the basis of either predominantly "outer sphere" proton relaxation (1, 2); slow exchange between water coordinated to the enzyme Cu^{+2} and bulk water (3); or, intrinsically small paramagnetic relaxation effects. We sought to enhance the paramagnetic relaxation due to the Cu^{+2} and therefore focussed our efforts on fluoride ion, as a novel ^{19}F NMR relaxation probe, in order to realize the following potential advantages over other probes--water, halide ions (4,5)--which have been used. First, the concentration of F^- can be varied independently, so

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[2]Abbreviations used: galactose oxidase, GOase; longitudinal relaxation rate, $1/T_1 = R_1$; transverse relaxation rate, $1/T_2 = R_2$.

that the paramagnetic relaxation rates can be increased and binding constants more readily determined from analyses of the concentration dependence of the relaxation rates. Second, since ^{19}F has nuclear spin quantum number I = 1/2, analysis of the relaxation mechanism would not be complicated by quadrupolar effects (6). Third, the relaxation enhancement of F^- bound to a paramagnetic center should be intrinsically large: there will be a contribution to dipolar relaxation not only from interaction between the ^{19}F nuclear spin and unpaired electrons in orbitals centered at the metal ion, but also a contribution from electron spin in orbitals centered at the fluorine (7). The latter contribution may, in fact, be larger than the former (8). Finally, the NMR sensitivity of ^{19}F is comparable to that of protons. The use of F^- may entail the following disadvantages: the presence of HF_2^- or HF at low pH; attack by F^- or HF on glass vessels; and strong outer sphere relaxation effects, due to hydrogen bonding between F^- and NH or OH protons in enzyme and substrate.

We report here preliminary results from a study on GOase which demonstrate the potential utility of F^- as a probe of paramagnetic metalloenzymes and which illustrate the precautions required. GOase catalyzes the conversion of primary alcohols to their corresponding aldehydes with the simultaneous reduction of O_2 to H_2O_2 (9). The paramagnetic center of GOase, a type II Cu^{+2} site (10), is apparently accessible to F^-, since relaxation enhancements of the order of 10 or more are produced by GOase at concentrations 0.2 mM or less, enhancements much larger than those due to comparable concentrations of apoenzyme.

Materials and Methods: Fluorine-19 relaxation measurements were made at 56.4 MHz via a Bruker-321s pulse spectrometer modified for field-frequency control. The ambient sample temperature was 26 $\pm$ 1.5°C. For solutions containing holoenzyme, the transverse relaxation rates, R_2, were obtained from free induction decays, since field inhomogeneity effects could be neglected. The longitudinal relaxation rates, R_1, were measured via the 180-τ-90 se-

quence (11). For the apoenzyme samples, R_2 values were obtained from Carr-Purcell pulse sequences, with the Gill-Meiboom modification (12), and R_1 values were derived from the half-wave triplet sequence (13).

The source of F^- was $KF \cdot 2H_2O$ (Matheson, Coleman and Bell). D(+)-galactose (SIGMA) was added to sample tubes as a solid which had been dried under vacuum and stored under dry N_2. In the galactose competition experiments, deoxygenation was effected by bubbling wet N_2 gas through all solutions before addition of the sugar; transfers were made under an atmosphere of wet N_2. KCN (Fisher) was the source of CN^-.

All solutions were maintained at a pH = 7.0± .05 by use of 0.050 M $NaH_2PO_4 \cdot H_2O$ (Fisher) and NaOH, and at an ionic strength in the range 0.97-1.06 M by the addition of K_2SO_4. Galactose oxidase was prepared following literature procedures (14,15). The concentration of enzyme in solution was derived from absorbance measurements at 280 nm. The specific activity of the enzyme was determined for some samples, both in the presence and absence of F^-, by means of a standard assay reaction (14). In all cases the specific activity of the enzyme was reduced by F^- to approximately 2/3 the original value. Upon exhaustive dialysis of the enzyme-fluoride sample against buffer, to rid the sample of F^-, the specific activity was restored to its original value.

Measurements of relaxation times were taken on samples contained in glass, Delrin or KEL-F sample tubes which had been washed with concentrated nitric acid. Buffer solutions were prepared from de-ionized, glass-distilled water; some buffer solutions were passed through a CHELEX 100 (Bio-Rad Laboratories) column before use. Both R_1 and R_2 values of the holoenzyme samples were essentially independent of sample tube material. The relaxation rates (especially R_2) of the aqueous F^- solutions with no holoenzyme present were strongly dependent upon tube type and method of buffer preparation. Since the apoenzyme readily picks up free Cu^{+2} from solution, all relaxation measurements involving apoenzyme used samples con-

TABLE I

Water Proton Relaxation Rate Changes Due to GOase[a]

System[b]	ΔR_1 (sec^{-1})	ΔR_2 (sec^{-1})
+ air	0.24	1.46
+ O_2 (saturated)	0.47	1.56
deoxy	0.28	1.70
deoxy + galactose[c]	0.28	1.64

[a] at 26°C and 60 MHz.

[b] concentration of GOase = 0.1 mM

[c] concentration of galactose = 0.4 M

TABLE II

Fluoride Ion Relaxation Rate Changes due to GOase[a]

F^- concentration[b] (M)	ΔR_1 (sec^{-1})	ΔR_2 (sec^{-1})
0.820 + apoenzyme	0.63	1.54
0.494 + holoenzyme	15.	59.
0.297 + holoenzyme	18.	48.

[a] at 26°C and 56.4 MHz

[b] relaxation rates for a GOase concentration = 0.1 mM, determined by interpolation of plots of ΔR vs. GOase concentration.

tained in KEL-F tubes and buffer solutions which had been passed through a CHELEX 100 column.

Results and Discussion: Tables I and II give relaxation rate changes, ΔR_1 and ΔR_2, due to GOase, for water protons and fluoride ions, respectively. Up to holoenzyme concentrations of about 0.2 mM, ΔR_1 for F^- varies linearly with GOase concentration at constant F^- concentration; at higher enzyme concentrations, ΔR_1 shows positive deviations from a linear plot, which feature may be due to aggregation of the enzyme. The plots of ΔR_2 vs. holoenzyme concentration are non-linear for GOase concentrations greater than about 0.1 mM, exhibiting much greater curvature than those for ΔR_1.

The much larger relaxation rate changes produced by the holoenzyme, compared to those due to the apoenzyme at similar concentrations, indicates that F^- binds at or near the Cu^{+2} site of the enzyme. The relative values of R_1 and R_2 in the presence of holoenzyme, along with the difference in

the concentration dependence of R_1 and R_2, imply that intermediate or slow exchange conditions (at the ambient temperature) limit the value of R_2 for F^-, whereas the fast exchange limit may be appropriate, even if only approximately so, for R_1. From the concentration dependence of ΔR_1 in the linear region, we have estimated an approximate value, $K = 1.2\ M^{-1}$, for the binding constant of F^- to GOase. In this analysis our assumption that only one F^- binding site per GOase molecule is present yields a good fit to R_1 in the linear range for GOase concentration.

The close agreement of the value of K thus determined with the value $K' = 6.8\ M^{-1}$, for the association constant of CuF^+ in aqueous solution (16) would be consistent with direct binding of F^- to enzyme Cu^{+2}. However, the value of $R_{1M} = 2 \times 10^5\ sec^{-1}$ for F^- bound to the enzyme (as determined from the analysis cited above) is at least an order of magnitude smaller than that which we have estimated for CuF^+ in aqueous solution from the data of Eisenstadt and Friedman (8). This last finding would suggest either that the binding of F^- to enzyme Cu^{+2} is indirect, i.e. enzyme bound F^- is in the second coordination sphere of Cu^{+2}, or that the CuF^+ moieity is strongly perturbed by interactions of F^- with groups adjacent to the Cu^{+2} site, possibly NH or OH groups which could hydrogen bond to F^-.

Experiments with F^- and CN^- as competitive binding agents show also that fluoride binds at or near the enzyme Cu^{+2}: in the presence of GOase, the enhanced F^- relaxation rates decrease as CN^- is added, up to a 3:1 molar ratio of CN^- to enzyme. ESR results (17) indicate that CN^- binds stoichiometrically to the enzyme Cu^{+2}, which result is consistent with that from our competition experiment and the assumption of a single binding site for F^- at the enzyme Cu^{+2}, if the binding constant for CN^- to enzyme Cu^{+2} is less than about $10^5\ M^{-1}$. This limit for the CN^----Cu^{+2} binding constant is not inconsistent with the ESR results.

Competition experiments between D(+)galactose, an enzyme substrate, and F^- have also been carried out. As shown in Fig. 1, when galactose is

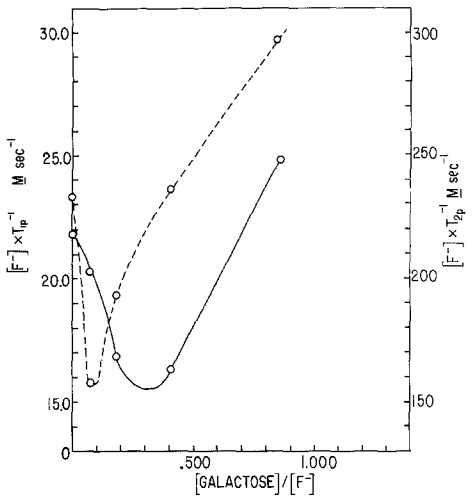

Fig. 1. Competition between D(+)galactose and F^- in the presence of [GOase] = 1.2×10^{-4} M. $T_{ip} = R_i(\text{holo}) - (\text{apo})$, i = 1 or 2, for equivalent concentrations. $[F^-] = 0.82$ M. ——: $[F^-] \cdot T_{1p}^{-1}$, ---: $[F^-] \cdot T_{2p}^{-1}$.

added to a solution in which F^- and GOase concentration is maintained constant, the fluoride relaxation rates (both R_1 and R_2) initially decrease and then increase on further addition of galactose; at a galactose to fluoride concentration ratio of about 1:2, or greater, the F^- relaxation rates are greater than in the absence of galactose. The addition of glucose, which is not a substrate for GOase, does not change the enhanced values of R_1 in the presence of holoenzyme, although a small decrease in the value of R_2 does occur. A detailed interpretation of these results awaits determination of binding constants for F^- and free galactose, but it is evident, in any case, that a ternary or higher order complex is formed in which F^- (and, presumably, galactose) are close to the paramagnetic center of the enzyme.

We are continuing more detailed studies of F^- as a probe of GOase, including variable temperature and competition experiments with other substrates of GOase. We are also initiating tests of F^- as a probe of other copper-containing enzymes. For azurin, preliminary F^- relaxation rate measurements show that R_1 increases sharply at azurin concentrations greater

than about 1×10^{-4} M; this behavior may be related to exchange limited relaxation (3).

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